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AMPHETAMINE METABOLISM IN CIRRHOTIC MICE

by

YIP WAN LEE Ⓒ

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The undersigned certify that they have read, and recommend
to the Faculty of Graduate Studies and Research, for acceptance, a
thesis entitled AMPHETAMINE METABOLISM IN CIRRHOTIC MICE submitted
by YIP WAN LEE in partial fulfilment of the requirements for the
degree of MASTER OF SCIENCE IN BIONUCLEONICS.

TO ESTELLA

ABSTRACT

The metabolism of amphetamine by control, phenobarbital (PB) treated and phenobarbital-carbon tetrachloride (PB-CCl$_4$) treated mice was studied. Cirrhosis of the liver was induced by the combined treatment of PB and CCl$_4$. The presence of the cirrhotic condition was confirmed by electron microscopic examination of hepatic tissue.

Using radioactive carbon-labelled amphetamine sulfate, the urinary and fecal excretion of the drug were measured. In 72 hours, 84.5% of the injected dose was excreted in the urine by the control group, 61.5% by the PB group and 72.3% by the PB-CCl$_4$ group. Corresponding fecal values were 1.2%, 2.0% and 2.4% respectively.

Metabolites were identified using analytical thin-layer chromatography (TLC), autoradiography and gas-liquid chromatography (GLC). The number of major metabolites detected was seven in the control group, four in the PB group and three in the PB-CCl$_4$. A common urinary constituent detected and confirmed was amphetamine. The presence of p-hydroxyamphetamine was also confirmed in the urine of the PB-CCl$_4$ group; this hydroxylated analogue was detected, but its presence not confirmed in the other two groups. Also detected in trace amounts, but not confirmed, in the PB-CCl$_4$ group were phenyl-acetone, benzoic acid and hippuric acid.

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INTRODUCTION

Carbon tetrachloride has enjoyed widespread use in agriculture and industries. Gleason et al. (118) listed 109 CCl_4- containing commercial preparations, among which are fumigants, insecticides, Christmas tree "bubble lights," tar removers, degreasers, liquid fire extinguishers, water-proofing compounds, solvents and thinners. To workers in industries employing the chlorinated hydrocarbon exposure is an occupational hazard. To users of such products, accidental intoxication, whether mild or acute, is totally unwarranted. Environmental pollution by the noxious agent causative of several clinical cases of intoxication has been reported (119).

Long known as a hepatotoxin, the effects of CCl_4 on various plasma and hepatic enzymes are well known. However, its action on the hepatic drug-metabolizing system of enzymes is only sparsely documented with the assumption that a decline in metabolic activity would be the general rule. It is the purpose of this research project to study the capacity of the liver to handle a foreign compound, amphetamine, in the face of CCl_4-induced liver damage.

SURVEY OF LITERATURE

I. Amphetamine Metabolism

The urinary excretion of amphetamine has been reported by many researchers (3, 8, 16, 36, 56). A summary of the excretion rates in different animal species is presented in Table 1:

Table 1: Rate of Urinary Excretion of Amphetamine in Different Species (36)

Species	Sex	Route of admin.	Dose	Optical isomer	% of dose excreted 1st day	2nd day	3rd day
man	M	or	5mg	(+)	65	18	4
rat	F	or	10mg/Kg	(+)	79	–	–
mouse	F	or	10mg/Kg	(+)	78	–	–
guinea-pig	F	ip	5mg/Kg	(+)	86	–	–
Rhesus monkey	F	or	0.66mg/Kg	(+)	58	–	12
Rabbit	F	or	10mg/Kg	$(\overset{+}{-})$	75	–	–
Greyhounds	F	ip	5mg/Kg	$(\overset{+}{-})$	75	–	–

M: male
F: female
or: oral administration
ip: intraperitoneal administration

The guinea-pig has exhibited the fastest elimination of the drug, and the monkey the slowest. Urinary pH's were not reported by the authors. The excretion of amphetamine, which is a weak base with a pKa of 9.93 (75) or 9.77 (73) was pH dependent (3, 8, 56). An acidic urine would favor the ionic species and its renal excretion, whereas an alkaline urine would enhance tubular re-absorption of the unionized base. On the other hand excretion of the metabolites, which are usually more water-soluble, would be less likely to be influenced.

p-Hydroxyamphetamine, because of its polar hydroxyl group, has a pKa
of 10.7 (75). Benzoic acid and hippuric acid have pKa's of 4.19 and
3.80 respectively (59).

Amphetamine has been reported to be metabolized along the
following pathways (Fig. 1):
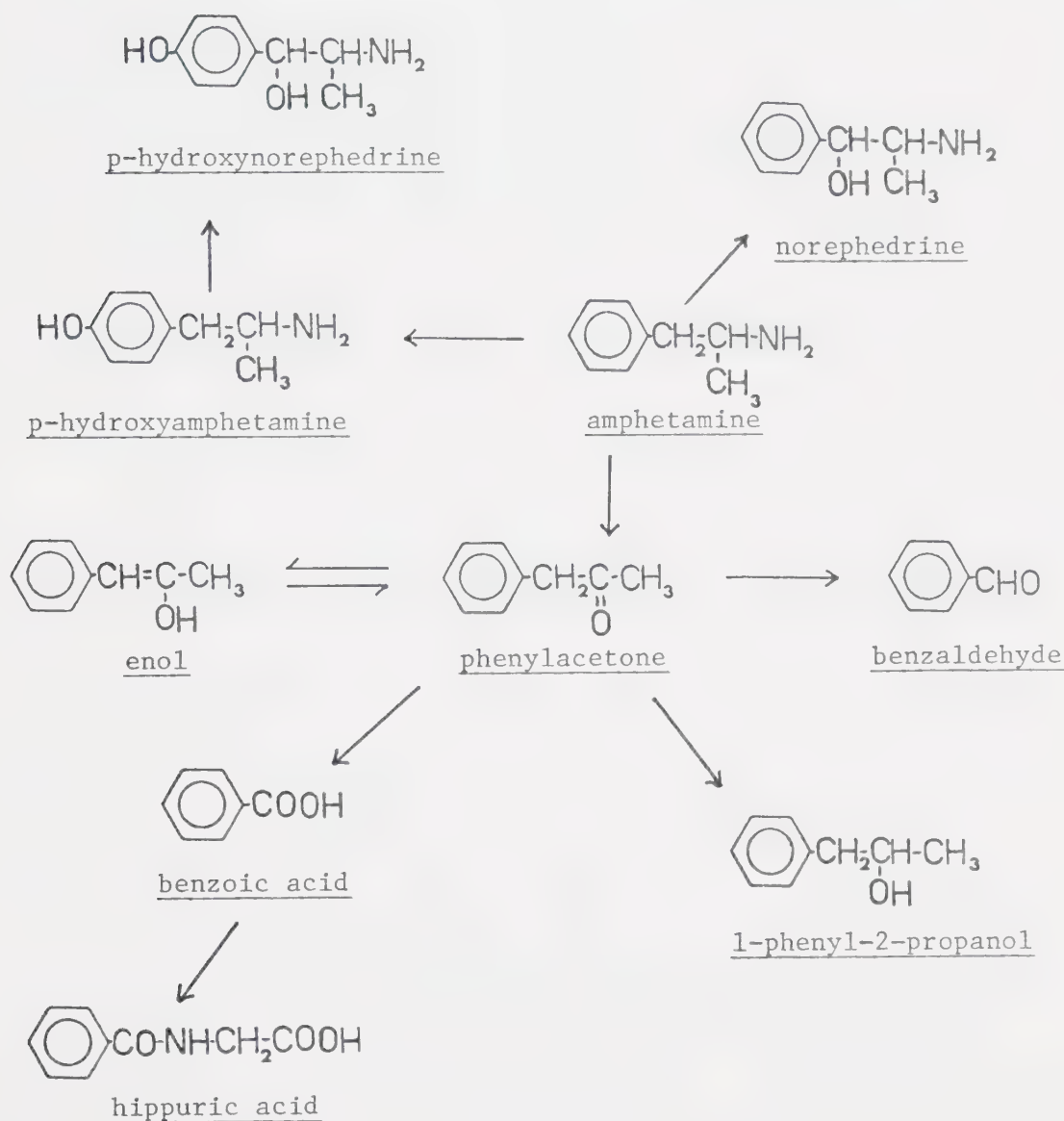
Fig. 1: Metabolic Pathways of Amphetamine

1. p-Hydroxylation of the phenyl ring led to the formation of p-hydroxyamphetamine which was excreted unchanged or as its conjugates.

2. β-Oxidation of the isopropyl side chain would yield the corresponding norephedrine or p-hydroxynoredphedrine.

3. Oxidative de-amination to the ketone which would tautomerize to the enol form. Oxidation of the ketone would produce benzoic acid; its reduction, an alcohol.

The p-position of the ring and the β-carbon of the isopropyl amine side chain were the major hydroxylating centres. p-Hydroxylation was non-stereospecific (51, 57) and was mainly a hepatic function. The p-hydroxyl derivative could then be excreted as such or as glucuronides, sulfates or acetates.

β-Hydroxylation has been shown to be a stereoselective step involving only the (+)-isomers (51, 56, 57, 123). In contrast with the p-hydroxylation process, this reaction took place mainly in the catecholamine-containing granules of the sympathetic nerves. The greater extensiveness of the metabolism of the dextro forms may partly explain the differential excretion rate for the two isomers. Eighteen percent less of the dextro isomer was excreted than the laevo during the first four hours after a dose of the racemate (57). The results were reproducible with either an acidic or alkaline urine (56). Other probable reasons for this favored excretion were selective uptake (56, 57) and a prolonged retention of the d-isomer (51, 56, 57).

Two different oxidative de-amination pathways have been postulated: α-C-oxidation (60, 95) and N-oxidation (7).

Hucker (60) and Parli et al. (95) suggested that hydroxylation of an imine intermediate was the most likely route to the oxime formation (Fig. 2). Another oxime precursor could be the N-hydroxylated form of a carbinolamine. Hydrolysis of the oxime would result in the loss of the basic moiety. The main drawback of the imine theory is that direct hydroxylation of the ketimine has yet to be demonstrated (60).
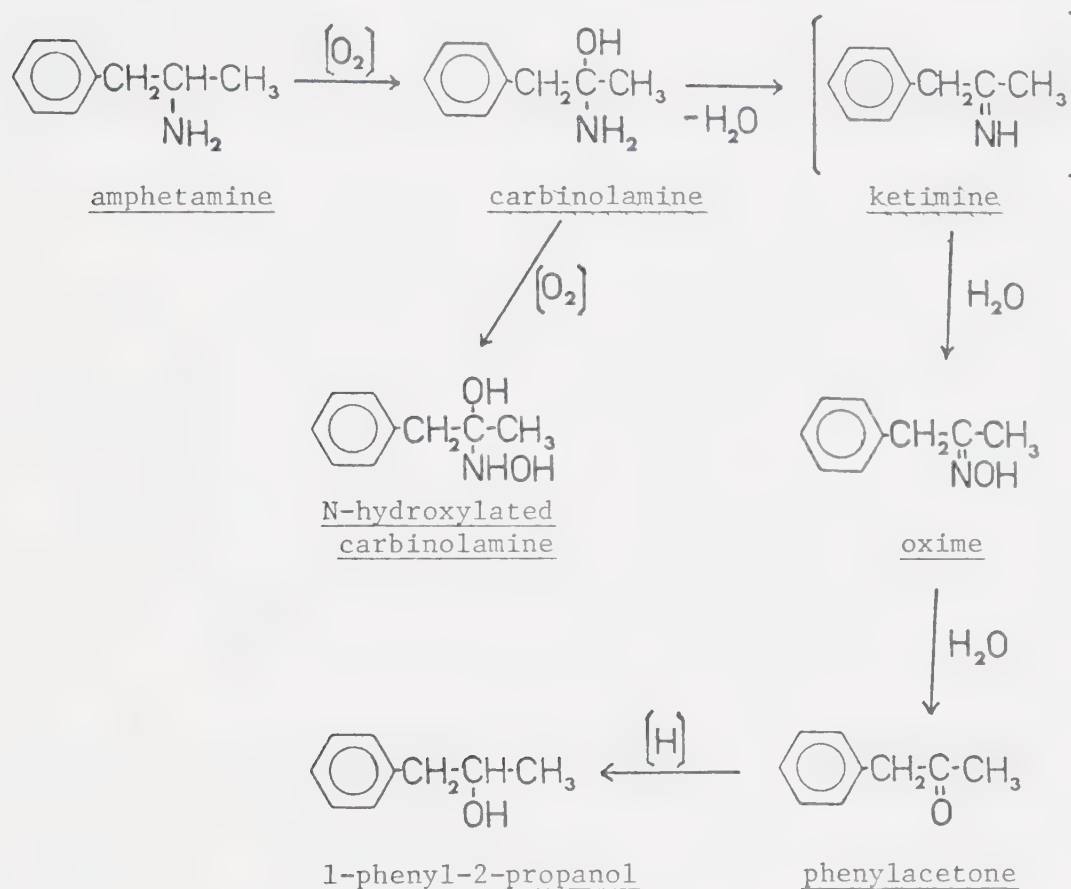
Fig. 2: Oxidative de-amination as proposed
by Hucker (60) and Parli et al. (95)

The reduction of the ketone to the secondary alcohol was stereospecific as only the (+) antipode was excreted (126).

Beckett and Al-sarraj (7) while not excluding the probability of an α-C-oxidation, argued that the major pathway to de-amination was

N-oxidation (Fig. 3), that the oximes and the ketone were products of chemical manipulations, and that hydroxylamine was not metabolized by microsomal enzymes.

Enzymatic oxidation on the nitrogen atom would yield a N-oxide which would be converted to the hydroxylamine by proton transfer. Acid hydrolysis of the latter would give phenylacetone, ammonia and oximes. Oximes could also be formed from alkaline hydrolysis and metabolic dehydrogenation of the hydroxylamine.
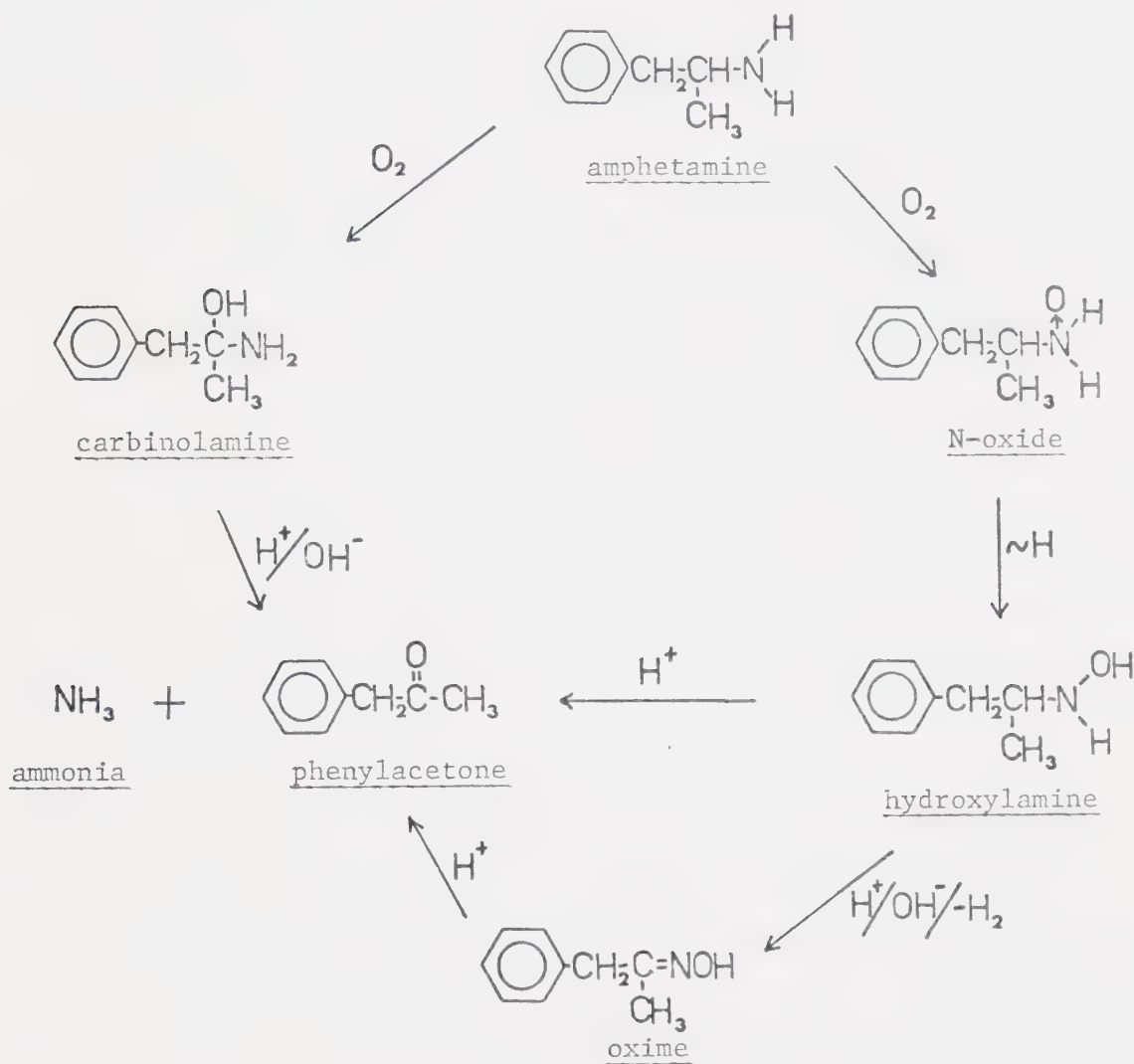
Fig. 3: Oxidative de-amination of amphetamine as proposed by Beckett & Al-sarraj (7)

Hucker had his doubts about this N-oxidation route (60). He had not been able to detect any hydroxylamine, which was believed to be only slowly metabolized or not at all.

The de-amination product, phenylacetone, could be further transformed, either chemically or enzymatically. The mechanism by which benzoic acid was formed from the ketone has remained obscure (125). The aldehyde has been said to be a chemical reduction product (7), whereas the secondary alcohol was due to metabolic reduction (7). The ketone undergoes an enol-keto tautomerism (Fig. 4). The enol content was reported to be almost 3% in methanolic solution (44).
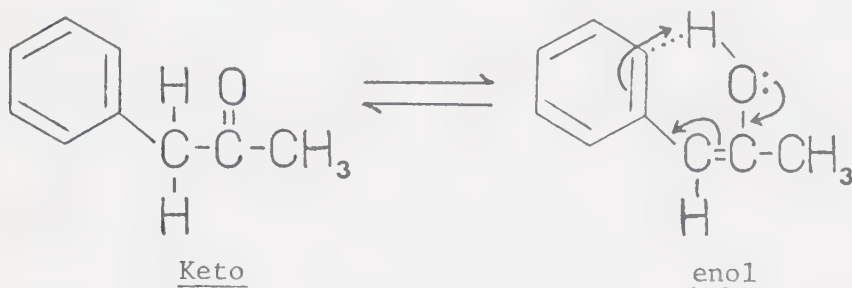
Fig. 4:   Enol-keto tautomerism of phenylacetone (10)

The proton involved is believed to be methylenic in origin. Stabilization energy would be supplied by the conjugation of the side chain double bond with the aromatic sextet (Fig. 4). The enol substituent is ortho and para directing. The partial negative charge at the ortho position permits intramolecular hydrogen bonding with the enolic hydrogen (Fig. 4). The enol was excreted as an oxygen sulfate.

A list of the reported metabolites is given in Table 2. Much interspecies variation has been found to exist as shown in the table. Amphetamine was shown to be incompletely metabolized by the guinea-pig,

Table 2: Metabolites of Amphetamine in Different Species

The value for each metabolite is expressed as a percentage of the excreted dose in 24 hours after dosing. + indicates the presence of the particular metabolite but the concentration of which is not reported.

Species	Sex	Route	Optical isomer	Amphetamine	p-hydroxy Amphetamine	benzoic acid	phenyl-acetone	1-phenyl-2-propanol	Norephendrine	p-hydroxy norephedrine	amphetamine hydroxylamine	phenylacetone oxime	Reference
Rat*	F	or	(+)	14	48	2			0.3	0.3			36
Rat	-	in vitro	(+)								+		7
Guinea-pig	F	ip	(+)	22	0	62							36
Guinea-pig	-	in vitro	(+)									+	9
Guinea-pig	-	in vitro	(+)								+		7
Mouse	F	or	(+)	33	14	31							36
Man	M	or	(+)	34	1	21	2				+		36
Man	M	or	(±)	19	3	28	1		2	0.4			16
Rhesus Monkey	F	or	(+)	18	6	35							36
Rabbit	F	or	(±)	4	6	25	22	8					36
Rabbit	-	in vitro	(+)				+	+					36, 61
Rabbit	-	in vitro	(+)								+		9, 60, 95
Greyhound	F	ip	(±)	30	6	28	1	1			+		7

* = a 48-hr. urine sample was investigated
f = female
m = male

or = oral administration
ip = intra-peritoneal administration

mouse, man and greyhound, in which more than 20% of the dose was excreted unchanged. On the other hand, metabolism was more extensive in the case of the rat, rhesus monkey and rabbit.

Aromatic hydroxylation reached its maximum in the rat, in which 48% of the administered dose was so converted. The mouse was a moderate p-hydroxylator with 14%. Man, rhesus monkey, rabbit and greyhound were poor in this capacity. The process appeared to be lacking in the guinea-pig. o-Hydroxylation has yet to be studied (125).

Benzoic acid was a major metabolite in all species except the rat. It made up 62% of the excreted dose in the guinea-pig, but accounted for only 2% in the rat.

The ketone and the alcohol were found mainly in the rabbit. Small quantities were also present in the urine of man and greyhound.

II. Carbon Tetrachloride Toxicity

CCl_4 had seen many industrial, medical and agricultural ap-
plications before its rapid, devastating effects on the host's liver
were discovered. Its widespread popularity has since been on a steady
decline. Many animals are known to be susceptible subjects of CCl_4
intoxication. Man, mice, rats, guinea-pigs, rabbits, cats, pigs, sheep
and monkeys are all affected. However, birds and chickens are quite
resistant, being able to tolerate about one hundred and ten times the
therapeutic dose for dogs without any hepatic lesions. Table 3
is a list of the major in vivo physiological and biochemical dis-
turbances in rats or mice following a single or multiple dose of the
toxic agent.

CCl_4 exhibits variable effects on different enzymes. Some of
the more commonly studied ones are shown in Table 4.

Ever since the recognition of the menance of CCl_4 in the late
nineteenth century, pathologists and physiologists have been probing
the manner by which the toxin operates, advancing different hypotheses
in the process (102). Some of the more important ones are outlined
below.

1. The phospholipid hypothesis (149)

It was believed that phosphilipids were the intercellular
transportation form of long-chain fatty acids, and that CCl_4 inter-
fered with hepatic phospholipid biosynthesis, diverting the acids to
metabolically inert triglycerides. The hypothesis was abandoned
when it was shown that such was not the manner of transport of fatty
acids.

Table 3: Physiological and biochemical lesions induced by CCl_4

Time (hr.)	Lesions	References
1/12	Lipid peroxidation	100
1/12	Incorporation of CCl_4 into microsomal lipids	101
0.5	Accumulation of triglycerides	76, 117
1	Alteration of endoplasmic reticulum (ER) structures	5, 133
1	Dispersion of polyribosomes	129
0.5 - 2	Degranulation and dilation of RER[a]	4, 5, 131
1 - 2	Depressed protein synthesis	118, 130
2 - 6	Disruption of lysosomes	2, 31
1 - 15	Mitochondrial degeneration	2, 4, 5, 17, 24 121, 137
5 - 12	centrilobular necrosis	24, 131, 145
6	mid-zonal necrosis	145
6	fibrogenesis	134
7	depletion of liver glycogen	115
18	nuclear injury	24, 133
40	Reduction of DNA,[b] RNA[c]	24
-	Increase in liver weight,	121
-	water & electrolyte imbalance	121
-	proliferation of SER[d]	133
-	Dissociation of 79S ribosomal units into 54S subunits	128
-	Increase in lysosomal units	133
-	Liver enzymes appear in plasma	34, 35, 108, 109
-	Proliferation of microbodies	5, 151

[a]RER: rough endoplasmic reticulum;

[b]DNA: deoxyribonucleic acid;

[c]RNA: ribonucleic acid;

[d]SER: smooth endoplasmic reticulum.

Table 4: Effect of carbon tetrachloride on enzymes

Enzymes	effect	references
Cytochrome P-450	decreased	22, 47, 127
Ethylmorphine demethylase	decreased	22, 53
Glucose-6-phosphatase	decreased	45
Aminopyrine demethylase	decreased	33, 47
Serum glutamic oxaloacetic transaminase	decreased	140
NADH-cytochrome C reductase	increased	106
Serum glutamic pyruvic transaminase	increased	63
NADPH-cytochrome C reductase	unaffected	47

2. The mitochondrial hypothesis (24)

The hypothesis stated that the toxic liver injury incurred by CCl_4 was a matter of mitochondrial degeneration. The idea soon fell into disfavor upon realization that the appearance of fatty liver preceeded mitochondrial damage by many hours.

3. The catecholamine hypothesis (18)

Both hepatic necrosis and fatty infiltration were thought to be due to a massive discharge of the sympathetic nervous system triggered by the action of CCl_4 on the central nervous system. Catecholamines diminished hepatic blood flow with associated centrilobular hypoxia and subsequent necrosis. The discharge also brought about an oversupply of fatty acids from the peripheral depots, accounting for the fatty liver.

However, the failure to produce hepatic necrosis in rats given large doses of epinephrine, norepinephrine, or mixtures of these catecholamines and the unsuccessful attempt to demonstrate an increase

in the flux of triglycerides through the plasma compartment during the period of triglyceride accumulation cast doubt upon the theory.

4. The solvent action hypothesis (150)

The hypothesis maintained that the toxicity of CCl_4 stemmed from its lipid solubility. What it failed to take into consideration were the relatively low toxicity exhibited by other lipid solvents and the lack of correlation between dose and the magnitude of response. Chloroform and diethylether are both lipid solvents with partition coefficients comparable to that of CCl_4. Yet these two solvents are only mildly to relatively non-toxic. The solvent action hypothesis was proven untenable upon the discovery that $10\mu M$ of CCl_4 per 100gm of body weight of rat was a sufficiently high enough dose to suppress the glucose-6-phosphatase activity in vivo (111) and that the same dose rate was capable of more than doubling the liver triglyceride content within six hours (103). The organ specificity of the toxin was the third point unaccounted for. Organs such as the brain, bone marrow, and the heart, where high concentration of the hydrocarbon accumulates, are relatively immune to the action of the chlorocarbon.

5. Toxic metabolite hypothesis (15)

This concept suggested that CCl_4 was metabolised in the liver to trichlormethyl ($CCl_3 \cdot$) and chlorine ($Cl \cdot$) free radicals which directly attack the various proteins and enzymes. However, no supportive evidence is available for such direct free radical attack.

6. The Lipid peroxidation hypothesis (48, 49, 102, 103, 104, 105)

This hypothesis has been found to be the most consistent with most in vitro and in vivo evidences. It states that lipid peroxidation

is the essential link between the initial metabolic homolytic cleavage of the carbon-chlorine bond and the sequence of epiphenomena that are so characteristic of CCl_4 poisoning.

Indisputable evidences in support of the requirement of metabolism in the expression of the toxicity of the haloalkane are now available (86, 112, 124). The ability to metabolise the poison has been shown to be an inherent property of susceptible animals. CCl_4 has been conclusively shown to be metabolised both in vivo and in vitro. Chloroform (15, 138), carbon dioxide (42, 82) and hexachloroethane (CCl_3CCl_3) (39, 138) were some of the identified metabolites. Some animals, such as the chicken (40) were insensitive to CCl_4. The lack of response has been associated with an inability to metabolise the chlorocarbon (148). In a similar case, new born rats, in which the metabolising system of enzymes is undeveloped, were also resistant to the toxin (29). Protection was also observed in rats on a low-protein diet (87) which diminished the level of enzymes.

Studies with enzyme inducers and inhibitors have strongly implied the role of metabolism. PB and 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)-ethane (DDT) are both metabolic inducers. Pre-treatment with the former (87, 99) and the latter (87) are known to hypersensitize the animal to a dose of CCl_4. On the other hand, CCl_4 is known to suppress metabolic activity. Its pretreatment has been shown to offer a transient protection against a normally lethal dose of the same poison (28, 47, 139). β-Diethylaminoethyl-diphenylpropylacetate (SKF 525A) is a competitive microsomal drug metabolism enzyme inhibitor. It has been found to partially prevent a CCl_4-induced depression of protein synthesis (119). It completely

inhibited CCl_4-induced loss of ethylmorphine demethylase (22). It completely prevented the in vitro (50) and almost completely the in vivo (99) peroxidative action of CCl_4.

Hepatic metabolism of foreign compounds usually involves the play of a mixed function oxidase system of enzymes located in the endoplasmic reticulum. Efforts to delineate the precise locus of metabolism have been centred around the flavoprotein NADPH-cytochrome P-450 reductase and on cytochrome P-450 itself. Most evidence seems to focus on cytochrome P-450 which is the site of metabolism of most foreign compounds.

Cogent evidence to support a direct involvement by cytochrome P-450 emerged from the observation of the remarkable resistance to the hepatotoxicity of CCl_4 exhibited in rat neonates (29) in which cytochrome P-450 activity was minimal at birth, whereas cytochrome C reductase, the measured flavoprotein, reached adult strength on the first day after birth. A comparable situation presented itself in the CCl_4-induced protection against its own toxic effects (28, 47, 139). The protective phase coincided with the depression phase of cytochrome P-450. The flavoprotein was virtually unaffected during the whole process.

The use of enzyme inducers has produced some very interesting results. PB is known to be a broad spectrum enzyme inducer. It will increase the metabolism of drugs that give a type I or type II binding spectra with cytochrome P-450. A type I binding spectrum is an indication of binding to the apoprotein. A type II spectrum will result from binding at the heme-iron moiety. CCl_4 will form a type I spectrum with cytochrome P-450 (20, 85) and its metabolism (42) and

toxicity (42, 71) have been found to be potentiated by PB. 3-methyl-cholanthrene (3-MC) is known as a limited microsomal enzyme inducer with potentiation power limited to type II compounds only. It will induce the formation of cytochrome P-488, reduce cytochrome P-450, but not affect the flavoprotein. As expected, it exerted a negative influence on most CCl_4 toxic effects (21). By reason of deduction, the active site of CCl_4 metabolism has been postulated to be the apoprotein of cytochrome P-450.

However, conflicting results were reported with 3,4-benzpyrene, a narrow spectrum enzyme inducer. The drug will induce the formation of cytochrome P-488, presumably from cytochrome P-450, and will have no effect on NADPH-cytochrome C reductase. Unlike 3-MC, it will enhance the toxicity of CCl_4 (97). Resolution of the difference should require further investigation.

If cytochrome P-450 is the catalytic centre for CCl_4 metabolism, compounds that are metabolised by the same enzyme would exhibit a competitive inhibition of its metabolism and toxicity. SKF 525A, aminopyrine, aniline, zoxazolamine, β-estradiol, deoxycorticosterone, progesterone, androstandione and pyrazole are all known in vitro antagonists of CCl_4 (104, 114). Complementary results from the in vivo studies with SKF 525A (99), pyrazole (27), hexobarbital, zoxazolamine and aniline (68) have substantiated the role of the hemoprotein. Competitive binding to cytochrome P-450 is the common mechanism of action in all cases.

In the mixed function oxidase system of enzymes, cytochrome P-450 and the flavoprotein form part of the electron transport train. Implication of an involvement by the system has been clearly demon-

strated by the action of ethylenediaminetetraacetate (EDTA) (49) which

completely prevented the pro-oxidant action of CCl_4 in a NADPH sup-

plemented system. A pronounced pro-oxidant property of CCl_4 was

evident when a normal flow of electrons through cytochrome P-450

was maintained (49). Extraneous electron acceptors, such as

ferricyanide or cytochrome C, when added to an in vitro system at the

beginning of incubation, are preferentially reduced. This short-

circuited flow of electrons results in an inhibition of the pro-

oxidant action of CCl_4 (49, 50) at the same time excluding any possible

role of the flavoprotein (50) whose duty in the system is solely as a

porter of electrons between NADPH and cytochrome P-450 where the

"lethal" cleavage takes place.

The toxicity of CCl_4 requires metabolism--the Cl_3C-Cl bond

cleavage. It has been described as "homolytic" (15) and "lethal" (104)

in which the bond rupture yields two free radicals: $\cdot CCl_3$ and $Cl\cdot$

(fig. 5). The cleavage can be either enzymatic or non-enzymatic. An

electric dipole exists in a carbon-halogen bond. The compound would

undergo homolytic fission if supplied with an external electric field

of vector opposite to that of the dipole.

$$Cl_3C-Cl \longrightarrow Cl_3C\cdot \quad + \quad Cl\cdot$$

Carbon	trichloro-	chlorine free
tetrachloride	methyl free	radical
	radical	
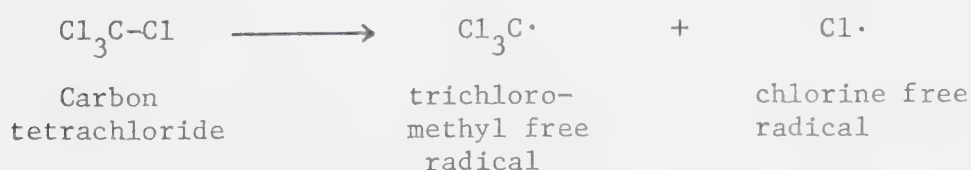
Fig. 5: The homolytic fission of carbon tetrachloride (15)

In view of the high dissociation energy required for uni-

molecular homolytic rupture of the carbon-chlorine bond at $37^\circ C.$, a

molecule-induced homolysis has been proposed (fig. 6) (147). A potentially toxic compound, A:B, upon attack by a pre-existing free radical, X·, would yield a new free radical, B·, with which the host system would be unable to cope.

$$A:B \ + \ X\cdot \ \longrightarrow \ X:A \ + \ B\cdot$$

Fig. 6: The facilitated homolysis (147)

One molecule of CCl_3·free radical per CCl_4 molecule could also be produced by the "electron capture" reaction (54):

$$CCl_4 \ + \ e \ \longrightarrow \ \cdot CCl_3 \ + \ Cl^-$$

Fig. 7: The "electron capture" reaction (54)

In a biological system, electron-transport reactions are adequate suppliers of the loosely bound electrons for such a reaction.

Recently, the possibility of the existence of a trichloro-methyl anion species, CCl_3^-, has arisen (138). The anion would be a product of two electron capture steps.

$$CCl_4 \ + \ e \ \longrightarrow \ \cdot CCl_3 \ + \ Cl^-$$
$$\cdot CCl_3 \ + \ e \ \longrightarrow \ CCl_3^-$$

Fig. 8: Production of trichloromethyl anion (138)

The evidence for homolytic cleavage is indirect. Homolytic cleavage would depend in part on the identification of hexachloroethane, (CCl_3CCl_3) (39, 138) and chloroform (15, 39, 138) as metabolites, and

partly on a study (66) of the comparative toxicities and dissociation energies of chloroform ($CHCl_3$), CCl_4 and bromotrichloromethane ($BrCCl_3$). A low bond dissociation energy would imply greater ease to cleave homolytically and more pronounced toxicity. The bond dissociation energies of the three compounds being $CHCl_3 \rangle CCl_4 \rangle BrCCl_3$ suggest a reverse order for their toxicities. $BrCCl_3$ has been found to be the most toxic and chloroform the most inert.

The protective effect of certain anti-oxidants (30, 41, 141) which act as free radical scavengers is certainly consistent with the homolytic concept of metabolism.

Contradicting results with vitamin E have been reported (23). The compound, while usually effective against lethality in male rats, was found to be deficient in its power in the female species. No explanation of the conflicting data has been offered.

The central idea of the lipoperoxidation hypothesis calls for an initial homolytic fission of the carbon-halogen bond followed by a free radical attack on the polyenoic side chains of the lipid centre of membrane lipoproteins. The peroxidative decomposition of the structural lipids is the link between the initial cleavage and the multitude of pathological events that follow.

Fig. 9 represents carbon 6 through 16 of arachidonic acid, a component structure of lecithin. The $CCl_3 \cdot$ free radical could abstract a bridge methylenic hydrogen from the fatty acid to form chloroform and a new fatty acid free radical. Alkylation would result in incorporation. The new fatty acid free radical resonates to a conjugated diene with characteristic absorption spectrum in the ultraviolet region (103). Paramagnetic molecular oxygen with unpaired
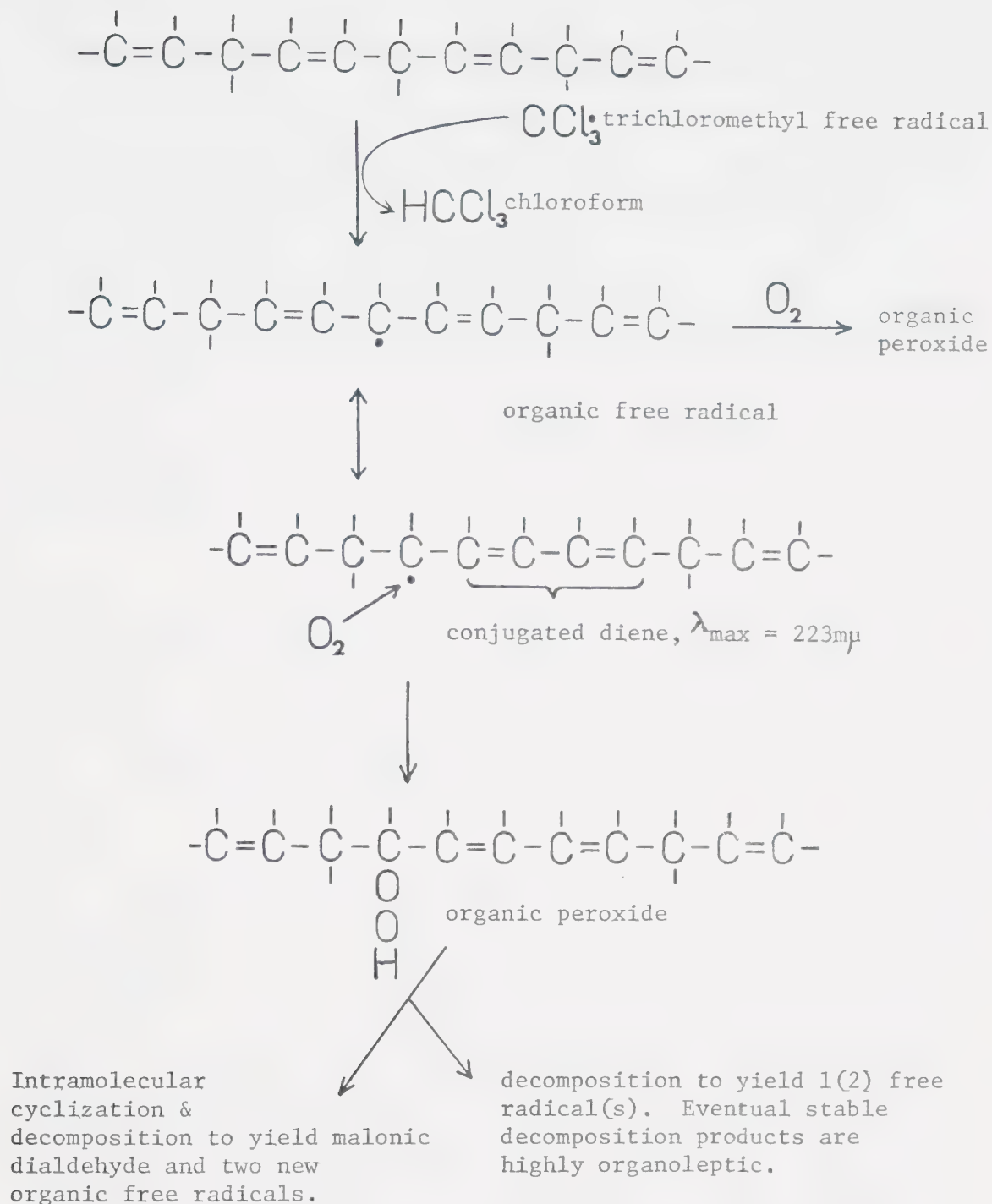
Fig. 9: Free radical initiated, autocatalytic peroxidation
of polyenoic long-chain fatty acids (101, 104)

electrons, itself a diradical, would then react readily with an organic free radical to form the corresponding peroxide. An organic peroxide is generally unstable and its decomposition products would be highly reactive free radicals. A snowballing free radical production would thus be initiated, and spread far beyond the original site of $CCl_3 \cdot$ free radical production.

Certain intracellular elements, such as the hemes of cytochromes (136) or redox pairs as in ferrous-ferric ions or manganous-manganic ions (6) could also catalyze the decomposition of hydroperoxides. During the process one new free radical would be produced:

$$ROOH + M^{3+} \longrightarrow ROO\cdot + M^{2+} + H^+$$
$$\text{hydroperoxide}$$
$$ROOH + M^{2+} \longrightarrow RO\cdot + M^{3+} + OH^-$$

Fig. 10: Metallic ions-catalysed decomposition
of hydroperoxide (6)

The lipid peroxidation hypothesis emphasizes the role of the peroxidative process. It also stipulates that peroxidation of structural lipids is not a normal cellular phenomenon. Living cells have various means to combat the process *in vivo*. Two poorly understood mechanisms are usually involved, the prevention of the onset of autoxidation and its interruption once initiated. Free radical scavengers and anti-oxidants such as vitamin E, and maybe superoxide dismutase (83) have been cited as examples of the former. Curtailment of the reaction could be achieved by reduced glutathione (25, 49).

The earliest substantiating evidence of the peroxidative aspect of the intoxication process depended on the efficacy of lipid

anti-oxidants such as vitamin E to suppress the lethal effects of CCl_4 in rats (23, 30). A conjugated double-bond absorption spectrum was later obtained as unequivocal proof of the presence of peroxidation (103). Peroxidation of the endoplasmic reticulum lipid was found to be sixty per cent complete within five minutes after administration of CCl_4 (100). The detection of abnormal branched-chain fatty acids within five minutes after CCl_4 administration (52) as end condensation products between $CCl_3 \cdot$ free radicals, or their oxy or peroxy derivatives, and lipid free radicals, contributed further support to the hypothesis.

Most cellular and subcellular organelles, such as endoplasmic reticulum and mitochondria, are membraneous in nature. Although the detailed chemical structures of lipoprotein membranes are still unknown, it has been assumed that normal structure and integral functions are inseparable. Any structural deviations from the norm would imply alteration or the loss of membrane and membrane-related functions. Lipid peroxidation has been associated with the change in activities of a variety of enzymic functions associated with lipoprotein membrane. Glucose-6-phosphatase (45), aminopyrine demethylase (48) and cytochrome P-450 (104) have been shown to decline rapidly in vitro as the microsomal lipids peroxidise. However, microsomal NADH cytochrome C reductase activity was reported to increase significantly (106). In another report (104), the enzyme level was reported to increase initially and then decline. Decreased activity was believed to be due to maximal lipid peroxidation. Alterations in the micellar state of the lipids due to less than complete peroxidation may be causative for the observed rise in activity (64).

Hepatic fatty infiltration is the most rapid in onset and best known of the CCl_4 poisoning symptoms. Advances in lipid biosynthesis have paved the way to the understanding of the genesis of this aberration. In the poisoned liver, the entire triglyceride synthetic route has been found to remain intact for at least twelve hours after intoxication (104). Accumulation of triglyceride in the liver is thought to be due to a blockade somewhere along the route of conversion of the preformed triglycerides to very low density lipoproteins and their final extrusion to the plasma (105). The involvement of lipid peroxidation in lipogenesis is only speculative at best. The coupling of preformed triglycerides with apoproteins is endoplasmic reticulum mediated and the process might be enzymatic. Any derangement of membrane structures could result in a disruption of coupling and exit to the plasma compartment (98, 107).

There was a rapid loss of cytochrome P-450 activity associated with CCl_4 administration (47, 53, 116, 127). EDTA pretreatment, by chelating metals of redox pairs prevented the destruction of the enzyme and lipid peroxidation (128). Cytochrome P-450 is believed to be "embedded" in microsomal lipids (92). The $CCl_3\cdot$free radical would initiate the formation of lipid peroxides and hydroperoxides; heme and heme-proteins would be capable of catalyzing the decomposition of the peroxidative products, being themselves rapidly destroyed during the process (91, 136).

Protein synthesis is a function of the RER. Inhibition of the process is known to be one of the early symptoms of CCl_4 intoxication (118). Administration of the toxin has appeared to result in widespread dislocation of ribonucleoprotein particles from ER mem-

brane surfaces (130, 131) and dispersion of 79S ribosomes into their

subunits (128). The dispersion of polyribosomes has also been ob-

served (129). Manifestation of any of the above reactions would

indicate a defect in protein synthesis. A loss of function due to

peroxidative degeneration of membrane lipids would be one causative

mechanism. A direct attack on membrane lipids, and thiol groups by

peroxy and hydroperoxy free radicals would be a second probability.

Thiol groups are thought to be essential in many steps along the

synthetic pathway (135). These essential groups are reported to be

particularly prone to free radical attack (146). The effectiveness

(38) of lipid peroxide and hydroperoxide to effect distant damages

makes this proposal most appealing. The large bulk of the protein

synthetic apparatus, involving as it does the many subcellular units

and enzymes, would demand a considerable space of separation between

itself and cytochrome P-450, the cleavage centre of the carbon-

chlorine bond. A direct attack would require a projection across the

space of separation; the chemistry of $CCl_3\cdot$ would make such an attack

most unlikely. In fact, a large number of hepatic functions are known

to be compromised during CCl_4 intoxication, and they cannot all occupy

the same locus, or be infinitely close together. The lipoperoxidation

hypothesis thus appears to best explain the pathogenesis of the defect.

While fatty infiltration, inhibition of protein synthesis and

other CCl_4-induced degenerative processes are poorly understood at

best, the knowledge of cell death and necrosis is completely wanting.

Cell death has usually been defined as the state in which the cellular

homeostasis is no longer maintained, and that the cell has lost its

integrity as a functional unit (78). In CCl_4-induced hepatic

necrosis, cell death has been dissociated from fatty accumulation.
Ethionine was found to be lipogenic but not necrogenic (102), anti-
histamines were shown to be effective protectors against necrosis but
not fatty infiltration (109) and thioacetamide produced hepatic cell
death without triglyceride accumulation (102). Cell death is com-
posite of many causes. According to the lipoperoxidation theory, the
ultimate death of the parenchymal cells is a result of peroxidative
destruction of cellular membranes.

EXPERIMENTAL METHODS AND RESULTS

I. Preparation of animals

Cirrhosis was induced according to the method of McLean et al. (88). Thirty male white mice (strain ALAS) were divided into three groups, all receiving the same stock laboratory chow. The first group of twelve animals served as the control. The second group of six animals, making up the phenobarbital group, had their drinking water replaced by sodium phenobarbital water (0.25 gm/ml.) for a total of thirty-seven days. The remaining twelve animals represented the PB-CC1$_4$. The latter group of animals were put on the same PB drinking water for seven days prior to and during the entire intoxication period. A desiccator of ten litres internal volume was used as an inhalation chamber. The mice, in groups of four, were placed inside the chamber. Using a syringe, 1 ml. of CC1$_4$ was introduced via a pipette onto a filter paper previously placed at the bottom of the desiccator and separated from the animals by a porcelain sieve. Exposure time was ten minutes daily for thirty days, at the end of which all animals again received tap water for drinking water.

II. Determination of radiochemical purity and specific activity of C-14-amphetamine sulfate
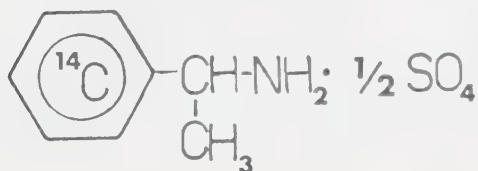
Fig. 11: [Ring-U-C-14]-amphetamine sulfate

Amphetamine, uniformly labelled with radioactive carbon-14 in the benzene ring, was obtained from Dr. A. Noujaim. The radiochemical purity of the compound was established by combined TLC-autoradiography. A little of the labelled compound was dissolved in a small volume of methanol and chromatographed on two silica-gel G[a] plates. Two different developing solvent systems (113), I (n-butanol: glacial acetic acid: water = 4:1:5 by volume) and II (95% ethanol: 25% aqueous ammonia = 4:1 by volume) were used. The developed plates were air-dried and sprayed with a 0.3% ninhydrin reagent[b] (143). Visualization of color was performed after heating at $110^{\circ}C$ for ten minutes. Reaction coloration for amphetamine was purplish red. Three spots were observed on both plates. The calculated R_f values in system I were 0, 0.38 and 0.43 and were 0, 0.59 and 0.67 in system II.

Autoradiography was carried out with Agfa-Gevaert X-ray films. Exposure time was for two weeks. The exposed films were developed at room temperature in Kodak X-ray developer for four minutes, washed briefly in water and then fixed in Kodak X-ray fixer for two minutes. Final washing required thirty minutes.

Four black spots were seen on each autoradiogram. In both instances, the high activity centres of the autoradiograms and the amino centres as revealed by spraying were coincident in their relative positions. The areas on the plates correspondent to the darkened areas of the autoradiograms were marked off by the use of a template and pin

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[a]Silica-gen G (Macherey, Nagel & Co.)

[b]Ninhydrin reagent (Fisher Scientific Co.)

and then scraped into scintillation vials to which Aquasol[R][a] (10 ml)
and distilled water (2.5 ml) were added. The addition of water causes
the fluor to gel, thereby suspending the silica-gel. The areas between
spots were divided into small sections and similarly treated. The vials
were scintillation counted in a Nuclear Chicago 720 Series Liquid
Scintillation Counter for forty minutes or 40,000 cpm in channel B.
An area of the developed plate away from the radioactivity was similarly
counted for determination of background activity. An average of 57
cpm background activity was found.

Table 5: Determination of the radiochemical purity of C-14
amphetamine sulfate

Solvent system I			Solvent system II		
R_f (Ninhydrin)	cpm -background	(% of total)[a]	R_f (Ninhydrin)	cpm -background	(% of total)[a]
	108			209	
	162			215	
	181		0.67	3,443	
	165			189	
0.43	5,673		0.59	463,190	(98.7)
	202			241	
0.38	348,956	(97.8)		211	
	189			1,450	
	1,379			141	
	106			165	
0	341		0	518	

[a]% of total = $\dfrac{\text{cpm-background}}{\Sigma(\text{cpm-background})}$ X 100

The two determinations averaged 98.2% purity. No further
purification steps were deemed warranted.

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[a]Aquasol[R], NEN, Boston, Mass.

The determination of specific activity was carried out in the following manner. The labelled compound (1.375 mg) was weighed out and dissolved in enough water to make 10 ml. Using micropipettes, 0.1 ml, 0.05 ml. and 0.02 ml. of the solution was withdrawn and counted in 10 ml. Aquasol® for ten minutes or 10^6 cpm. C-14-hexadecane[a] with a specific activity of 1.719 x 10^6 dpm/ml was used as a standard.

Table 6: Determination of specific activity of C-14-amphetamine sulfate

	(cpm)	% Eff.	Specific Activity (dpm/mg)	(Ci/mg)
A	146,245	90.69	11,727,864	(5.33)
B	75,443	91.51	11,991,678	(5.45)
C	30,195	91.72	11,971,375	(5.44)

Average specific activity = 11,896,972 dpm/mg
= 5.41 Ci/mg

III. Determination of counting efficiency of urinary and fecal samples

Six control mice were housed in separate cages which consisted of siliconized[b] 600 ml. beakers with double layers of siliconized[b] wire mesh at the bottom. The wire layer was of quarter inch wire mesh and the lower layer consisted of fly screen. Water was allowed ad libitum but food was withheld for the duration of the experiment. Urine and feces were collected for twenty-four hours. The volume of urine was adjusted to 10 ml. with distilled water. Urinary pH's were determined with narrow range pH paper (Whatman BDH).

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[a]Packard Instruments, Downers Grove, Ill.

[b]Apparatus was dipped in a 5% silicone solution in carbon tetrachloride and then dried at 400^oC. for six hours.

Table 7: Urinary pH and body weight for the control group

Mice	Weight (gm)	pH
1	36	9
2	34	9 - 9.5
3	32	9.5
4	34	8 - 8.5
5	35	9.5 - 10
6	36	9.5

Seven determinations of background activity were made by scintillation counting of 1 ml. of urine in 10 ml. Aquasol®. The average background was found to be 50 cpm. One ml. of standard C-14-toluene[a] (specific activity 4.26×10^5 dpm/ml) was diluted to 100 ml. with Aquasol®. 10 ml. of the resultant standard was pipetted into each of seven vials. To the first vial was added 1 ml. of Aquasol®. To the remaining six vials an addition of 1 ml. of urine was made.

Table 8: Determination of counting efficiency in urine-quenched samples

Sample	Urine (ml)	cpm	B/A[a]	% efficiency[b]
1	1	26,380	55.13	61.81
2	1	26,046	54.84	61.02
3	1	26,938	53.03	63.12
4	1	26,683	54.31	62.52
5	1	25,232	58.93	59.11
6	1	26,180	55.96	61.34
		Average	55.37	61.49

[a] B/A Channel ratio = Channel B/Channel A x 100.
[b] % efficiency = (cpm - background)$/4.26 \times 10^4$ x 100.

[a] C-14-toluene standard, NEN, Boston, Mass.

Fecal background activity was determined by first digesting and bleaching varying amounts of feces with 2.5 ml. of 30% hydrogen peroxide followed by ultrasonication. The vials were allowed to sit for twenty-four hours. The digested feces were suspended in 10 ml. Aquasol®. Counting was for forty minutes or 40,000 cpm in channel B.

Two levels of background activity were found. Samples smaller than 0.24 gm had an average background of 58 cpm, and samples larger than 0.24 gm had an average of 211 cpm.

A quench correction curve (Figure 12) was constructed by visual curve fitting to a plot of the percentage efficiencies versus the isotope channel ratios for vials containing differing quantities of feces and known amounts of radioactivity. The samples were treated as described for the blanks.

IV. Excretion of C-14-amphetamine sulfate

Three days after returning the mice to tap (drinking) water, six animals from each group were selected for metabolic experiments. A dose of 10 mg/kg body weight in 0.5 ml. distilled water was given intraperitoneally. Experimental conditions were similar to those used in the determination of counting efficiency. Cages were changed every twenty-four hours for seventy-two hours. The urine and feces were separated and scintillation counted in the manner previously de-scribed. The remaining urine was freeze-dried for identification of probable metabolites. The total weight of the feces was determined and a portion was treated as described for the fecal blanks.
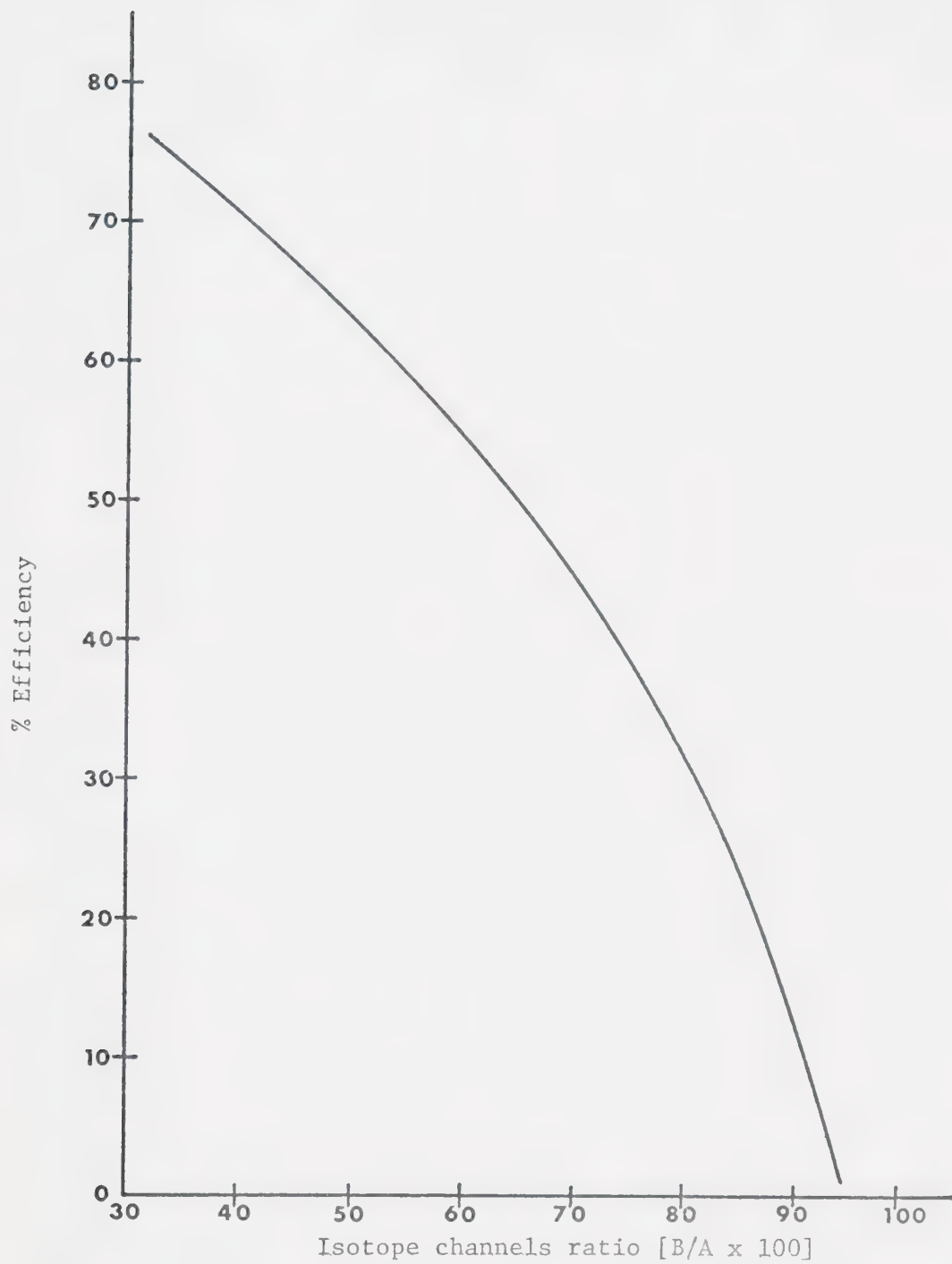
Figure 12:   Sample channels ratio quench correction curve for ^{14}C in feces

Table 9: Urinary Excretion of C-14-Radioactivity
(n=6 unless otherwise indicated)

Group (dose in dpm)	Mean urinary pH ± S.D.			Mean % dose excreted ± S.D.		
	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
Control[a] (3,711,855)	6.70 ± 0.27	6.45 ± 0.27	6.05 ± 0.27	82.73 ± 10.42	1.53 ± 1.34	0.27 ± 0.06
PB (3,580,988)	6.42 ± 0.26	6.42 ± 0.38	6.54 ± 0.33	60.30 ± 7.58	0.71 ± 0.50	0.49 ± 0.14
PB-CCl$_4$ (3,117,007)	6.63 ± 1.66	6.33 ± 0.38	6.17 ± 0.58	71.24 ± 3.21	0.47 ± 0.22	0.55 ± 0.20

[a] n = 5

Table 10: Fecal Elimination of C-14-Radioactivity
(n = 6 unless otherwise indicated)

Group (dose in dpm)	Mean % fecal elimination $\pm$ S.D.		
	24 hr.	48 hr.	72 hr.
Control (3,711,855)	0.86 ± 0.96^a	0.21 ± 0.07^a	0.11 ± 0.07^b
PB (3,580,988)	1.88 ± 2.95	0.16 ± 0.10	0.04 ± 0.02
PB-CCl$_4$ (3,117,007)	2.41 ± 1.69	0.04 ± 0.02	0

^{a} n = 5

^{b} n = 4

Table 11: Calculated t values of Excretion data

	Urinary excretion			Fecal Excretion		
	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
Control/PB	4.14^a	1.40	3.23^a	0.74	0.89	2.41^a
Control/PB-CCl$_4$	2.58^a	1.92	3.04^a	1.81	5.80^a	3.74^a
PB/PB-CCl$_4$	3.26^a	1.08	0.61	0.38	3.09^a	2.70^a

^{a} The difference is statistically significant at the 95% confidence level.

The twenty-four hours urinary excretion data of the three groups were found to be statistically different. However the differences were insignificant between the 48 hours samples. Among the 72 hours samples, differences between the control and the other two groups were again highly significant, while excretion by the PB group was not unlike that by the PB-CCl$_4$ group.

The situation was somewhat different in fecal excretion. All 24 hours data were found to be similar. The differences between the control and PB–CCl$_4$ groups, and between the PB and PB–CCl$_4$ groups were highly significant among the 48 hours samples. Significance was maintained in the 72 hours samples but at a reduced level. In addition, the 72 hours excretion data of the control and PB groups were also statistically different.

V.   Identification of metabolites

To aid in the identification of probable metabolites, two analytical methods, co-chromatography–autoradiography and GLC were employed.

The freeze-dried urine was dissolved in a little methanol and spotted on a 0.25 mm. silica-gel G thin-layer chromatography plate which was developed in solvent system I. Location of the various compounds was achieved by spraying with a 0.05% (weight by volume) alcoholic solution of bromophenol blue (93). The color reaction of the indicator with the various standards are given in Table 12.

Autoradiographic exposure time varied from one week for the 24 hours samples to two weeks for the 48 and 72 hours samples. Development time was four minutes for the 24 hours samples and eight minutes for the others. This was followed by one minute of washing and ten minutes of fixation and finally thirty minutes of washing. The position of each spot on the autoradiogram was traced out on the original plate. All the spots and the areas between them were scraped out and counted as described earlier.

Table 12: TLC R_f Values and Color Reactions of Amphetamine and Some of
Its Metabolites with Bromophenol Blue

Compounds	Mean R_f $\pm$ S.D.	Color Reaction
Amphetamine sulfate	0.55 ± 0.03	Blue
Amphetamine	0.41 ± 0.03	Blue
p-Hydroxyamphetamine hydrobromide	0.55 ± 0.05	Blue
p-Hydroxyamphetamine	0.29 ± 0.06	blue
N-Acetylamphetamine	0.76 ± 0.04	Blue
Benzoic acid	0.78 ± 0.05	Yellow
p-Hydroxybenzoic acid	0.73 ± 0.06	Yellow
Hippuric acid	0.61 ± 0.06	Yellow with an outer ring of blue

Table 13: R_f values and relative concentrations of the 24 hr.
control urinary metabolites

Radioactive urine			Standards	
Spot	R_f	%	Compound	R_f
1	0.81	1.85	Benzoic acid	0.84
2	0.74	33.28	p-hydroxybenzoic	
3	0.63	37.69	acid	0.80
4	0.56	1.49	N-acetyl amphetamine	0.79
5	0.52	8.58	hippuric acid	0.72
6	0.45	8.47	p-hydroxyamphetamine	0.60
7	0.16	4.61	amphetamine	0.58

Table 14: R_f values and relative concentrations of the 24 hr.
PB-treated urinary metabolites

Radioactive urine			Standards	
Spot	R_f	%	Compound	R_f
1	0.73	12.40	Benzoic acid	0.78
2	0.64	25.87	N-acetyl amphetamine	0.72
3	0.54	51.02	p-hydroxybenzoic	
4	0.42	3.22	acid	0.66
			hippuric acid	0.65
			amphetamine	0.56
			p-hydroxyamphetamine	0.54

Table 15: R_f values and relative concentrations of the 24 hr.
PB-CCl$_4$-treated urinary metabolites

| Radioactive urine | | | Standards | |
Spot	R_f	%	Compound	R_f
1	0.73	21.50	N-acetyl amphetamine	0.78
2	0.63	9.94	Benzoic acid	0.76
3	0.55	50.81	p-hydroxybenzoic acid	0.72
			hippuric acid	0.64
			amphetamine	0.52
			p-hydroxyamphetamine	0.51

Seven major metabolites were detected by autoradiography in
the control urine. In the PB-treated animals, the number decreased
to 4, and in the PB-CCl$_4$ group only 3 were observed. Tentatively
identified as common occurrences were amphetamine, N-acetylamphetamine
and hippuric acid.

To assist in the further identification of metabolites, the
following technique was employed. The freeze-dried urine samples
were pooled and extracted four times with 3 ml. of methanol. After
removal of the solvent the residue was dissolved in 1 ml. of Sørensen's
phosphate buffer (pH 7.4) (43). The buffered solution was divided in-
to 2 portions: control and enzyme hydrolysis. Incubation was carried
out for one hour at 37° C. β-Glucuronidase was added at the start
and again at time 0.5 hour. The pH of the incubation mixture was
adjusted to 10 and then 14. Each pH adjustment was followed by four
3-ml. ether extractions. The combined extracts were reduced to 0.5
ml. in a water bath maintained between 40 to 45° C. and under nitrogen.
The pH of the remaining aqueous solution was adjusted to 1. The ex-
traction and concentration procedures were repeated. Analysis of the
ethereal extracts was by TLC, autoradiography and GLC. Methylation

of carboxylic and phenolic functions prior to GLC was achieved by
the use of diazomethane (CH_2N_2) in ether.

The following GLC conditions were used:

    Instrument: Hewlett Packard 5700A Gas Chromatograph
    Detector: Dual flame ionization detectors
    Stationary phase: 3% OV225 on Chromasorb W 80 – 100 mesh
    Gas flow rate: Helium 60 ml./min.
                    Hydrogen 40 ml./min.
                    Air 300 ml./min.
    Column: Glass column O.D. 1/4 inch. Length 6 feet.

The GLC was operated isothermally. The standard amphetamine
free base had a retention time of 3.2 minutes at 115°C. p-Hydroxy-
amphetamine can be detected at 180°C. with a standard retention time
of 6.1 minutes. Their presence was confirmed by a peak augmentation
method with the standard compound.

Table 16: R_f values of 24 hr. control, enzyme-hydrolyzed
            urinary metabolites

| | Radioactive urine | | | | Standards | |
| | TLC | | Autoradiogram | | | |
	Spot	R_f	Spot	R_f	Compound	R_f
	1	0.71	1	0.62	phenylacetone	0.76
	2	0.36	2	0.49	hippuric acid	0.60
Basic	3	0.00	3	0.34	amphetamine	0.38
fraction			4	0.22	p-hydroxy-	
			5	0.13	amphetamine	0.22
	1	0.51	1	0.64	phenylacetone	0.85
	2	0.19	2	0.52	benzoic acid	0.84
Acidic	3	0.07	3	0.33	p-hydroxy-	
fractions	4	0.00			benzoic acid	0.80
					hippuric acid	0.74

Table 17: R_f values of 24 hr. PB-treated, basic urinary metabolites

| | Radioactive urine | | | | Standards | |
| | TLC | | Autoradiogram | | | |
	Spot	R_f	Spot	R_f	Compound	R_f
Control incubation	1	0.45	1	0.64	phenylacetone	0.71
			2	0.44	hippuric acid	0.55
					amphetamine	0.41
					p-hydroxy-amphetamine	0.26
Enzyme-hydrolyzed	1	0.40	1	0.59	phenylacetone	0.74
			2	0.38	hippuric acid	0.57
					amphetamine	0.41
					p-hydroxy-amphetamine	0.35

Table 18: R_f values of 24 hr. PB-treated, acidic urinary metabolites

| | Radioactive urine | | | | Standards | |
| | TLC | | Autoradiogram | | | |
	Spot	R_f	Spot	R_f	Compound	R_f
Control incubation	1	0.54	1	0.64	phenylacetone	0.76
	2	0.35	2	0.52	benzoic acid	0.69
	3	0.27	3	0.33	p-hydroxy-benzoic acid	0.64
					hippuric acid	0.54
Enzyme-hydrolyzed	1	0.52	1	0.50	phenylacetone	0.80
	2	0.40	2	0.39	benzoic acid	0.75
	3	0.28	3	0.30	p-hydroxy-benzoic acid	0.71
					hippuric acid	0.61

Table 19: R_f values of 24 hr. PB–CCl$_4$-treated, basic urinary metabolites

		Radioactive urine			Standards	
		TLC	Autoradiogram			
	Spot	R_f	Spot	R_f	Compound	R_f
Control incubation	1	0.75	1	0.74	phenylacetone	0.78
	2	0.68	2	0.66	hippuric acid	0.57
	3	0.47	3	0.47	amphetamine	0.45
					p-hydroxy- amphetamine	0.36
Enzyme- hydrolyzed	1	0.68	1	0.69	phenylacetone	0.78
	2	0.46	2	0.47	hippuric acid	0.57
					amphetamine	0.42
					p-hydroxy- amphetamine	0.28

Table 20: R_f values of 24 hr. PB–CCl$_4$-treated, acidic urinary metabolites

		Radioactive urine			Standards	
		TLC	Autoradiogram			
	Spot	R_f	Spot	R_f	Compound	R_f
Control incubation	1	0.55	1	0.53	phenylacetone	0.82
	2	0.40	2	0.39	benzoic acid	0.79
					p-hydroxy- benzoic acid	0.74
					hippuric acid	0.60
Enzyme- hydrolyzed	1	0.49	1	0.48	phenylacetone	0.81
	2	0.40	2	0.39	benzoic acid	0.78
					p-hydroxy- benzoic acid	0.74
					hippuric acid	0.61

Amphetamine was detected in all urine samples. At the same attenuation factor, the observed amphetamine peaks from the hydrolyzed samples were very much larger than those from the control incubation. A large portion of the amphetamine which was not metabolised was probably excreted in a conjugated form. Enzymic hydrolysis makes available the free base for ether extraction.

p-Hydroxyamphetamine was detected in the PB-CCl$_4$ treated samples. However, its intensity was not much different between the control incubation and the enzyme-hydrolyzed samples.

VI. Electron Microscopy

To evaluate the status of hepatic and renal involvement in PB-CCl$_4$ intoxication, the ultrastructures of the two tissues were examined. Specimens from all three groups were studied for comparison. The following procedures were used.

After ethereal anesthesia, the tissue was exposed and slightly flushed with ice-cold 2.5% glutaraldehyde (Fisher) in pH 7.4 Sørensen's phosphate buffer (43). A thin section was removed and minced into 1 mm. cubes with sharp scissors in a small vial in the presence of the glutaraldehyde solution. The spent fixative was replaced by fresh solution in which the tissue was allowed to remain for 2 hrs. All preparations were carried out in an ice-bath. After 2 hrs. the tissue was washed four times with the same cold buffer before post-fixation with ice-cold 1% osmium tetroxide (Fisher) in Sørensen's phosphate buffer for 1 hr.

The specimens were washed in cold distilled water and dehydrated in ethanol at room temperature. A graded series of ethyl

alcohol dilutions was used: 50%, 70%, 80%, 95% and 98%. Five to ten minutes were allowed in each bath with three changes of the absolute alcohol. Clearing with propylene oxide (Fisher) for 1 hr. completed the process. The clearing agent was replaced, at the end of the hour, by a 1:1 mixture of propylene oxide and the embedding medium, Araldite.[a] The specimens were allowed to remain in this mixture overnight before passing on to the pure embedding medium for one day.

The impregnated specimens were placed in BEEM® capsules (Fisher) and covered with fresh embedding medium. Final polymerization of the plastic was obtained by heating in an oven at 60°C. for 48 hrs. after which the blocks were ready for trimming and sectioning.

Copper grids with a 200 mesh were used. These were cleaned in glacial acetic acid and rinsed with distilled water and acetone. The grids were coated on the dull side with a thin film of Formvar® (Ladd) according to the method described by Pease (96), using a 0.25% chloroform solution of the plastic.

The blocks were sectioned with a Sorvall Porter-Blum Ultramicrotome MT-2. Glass knives were prepared from LKB glass strip with a LKB Knifemaker Type 7801B. Only silver sections, with thickness between 600 and 900 $\overset{o}{A}$, were accepted. The sections were floated in a 10% aqueous acetone bath and flattened with xylene vapor.

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[a]Araldite:
    Araldite resin 502 (polysciences, Inc.) 1 part
    Dodecenyl succinic anhydride
                  (Polysciences, Inc.) 1 part
  DMP-30 (Polysciences, Inc.)   2%

The sections were double-stained with 5% aqueous uranyl

acetate (96) and Reynolds' lead citrate (110) both of which were

force-filtered through two successive Millipore filters (Millipore

Corp.) before use. Staining time, identical in both cases, was

fifteen minutes. The sections were viewed in a Philips Electron

Microscope Model 300.

Plates 1 to 14 are electron micrographs illustrating some of
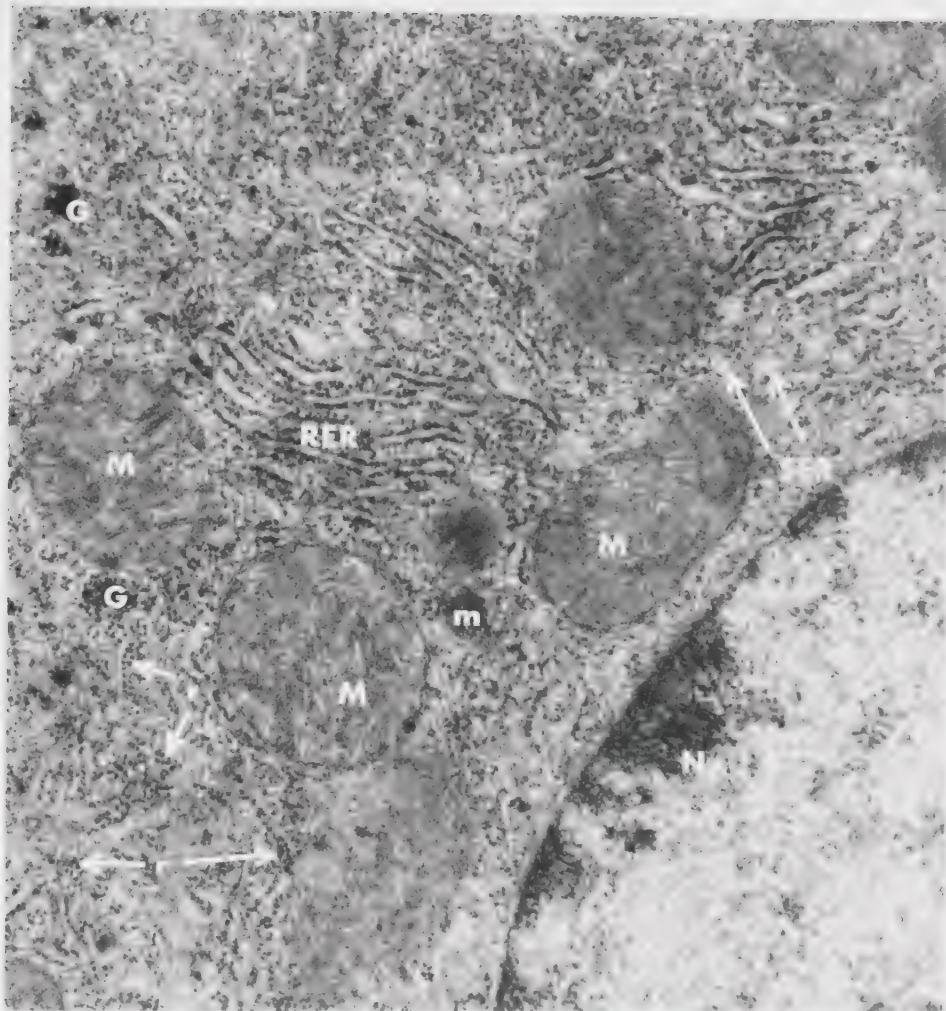
the actions of PB and PB-CC1$_4$ on hepatic and renal cellular structures.

Plate 1: A normal mouse hepatocyte. X32,300.

        Prominent in Plate 1 are the mitochondria (M) with their
moderately electron-dense contents and double membranes. They are in
close association with the parallel arrays of RER. Also seen are a
portion of a nucleus (Nu), some microbodies (m), glycogen granules (G),
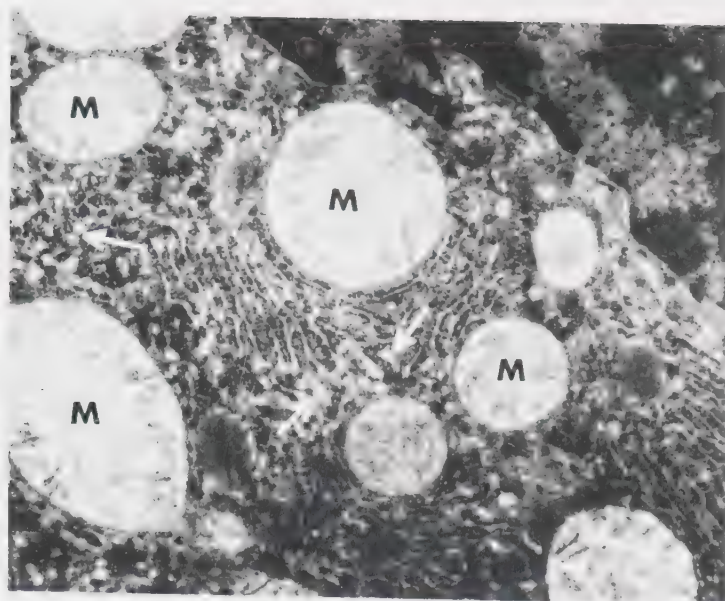free ribosomal units (r) and SER (arrows).
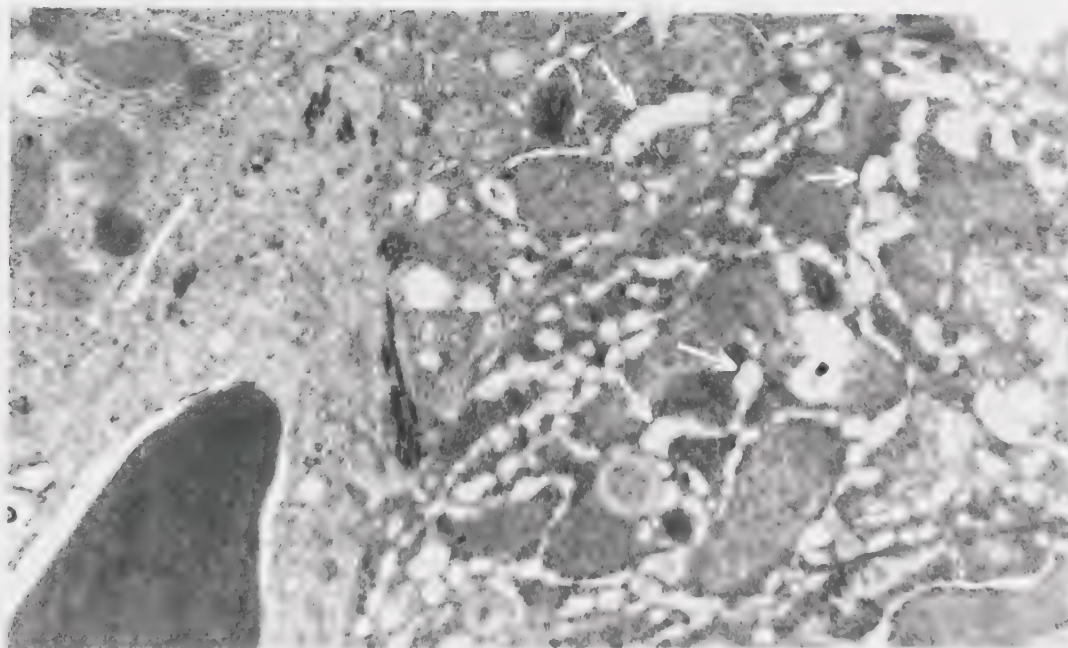
Plate 2:   PB-treated liver.   X 20,000.

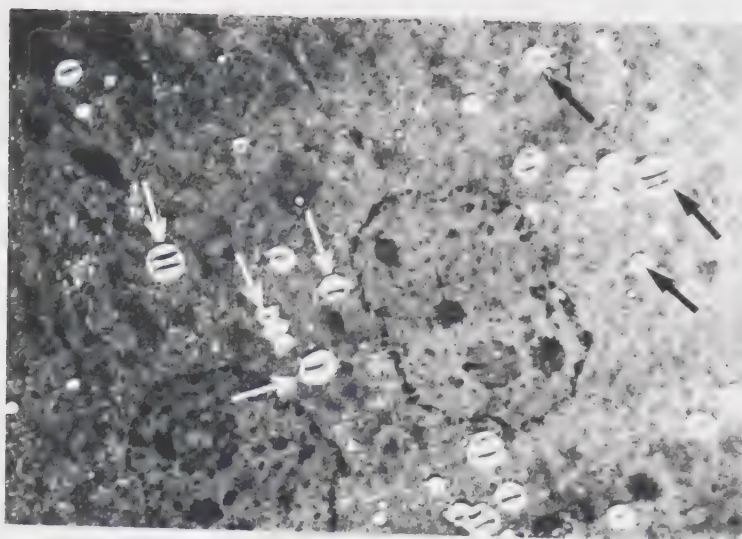Plate 3:   PB-treated liver.   X 17,200.
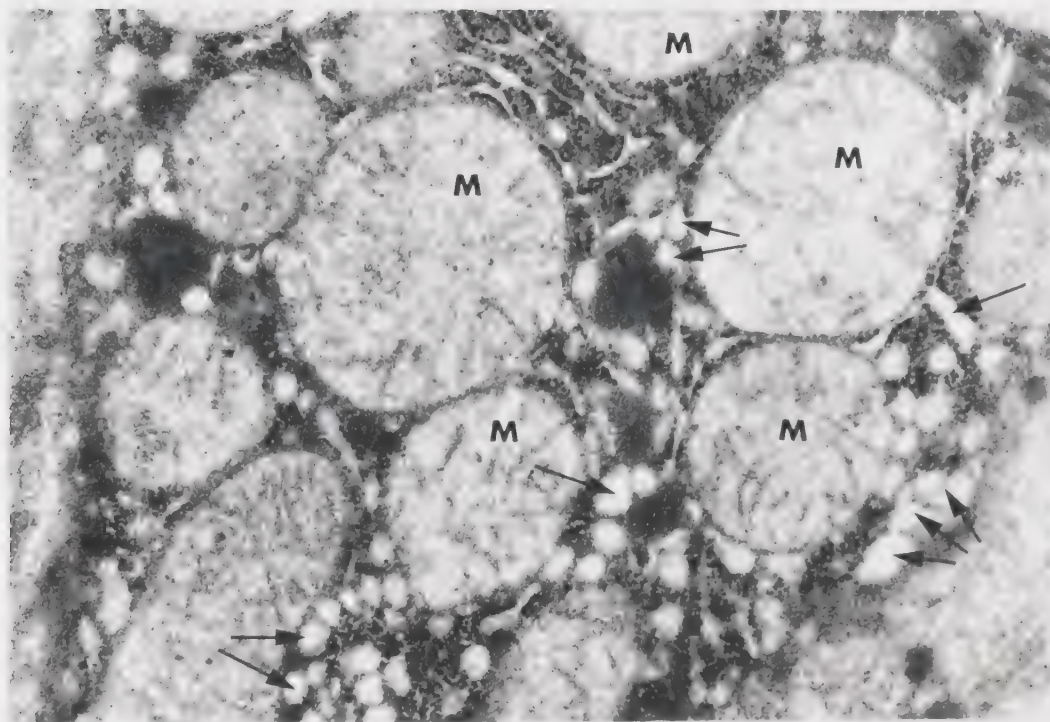
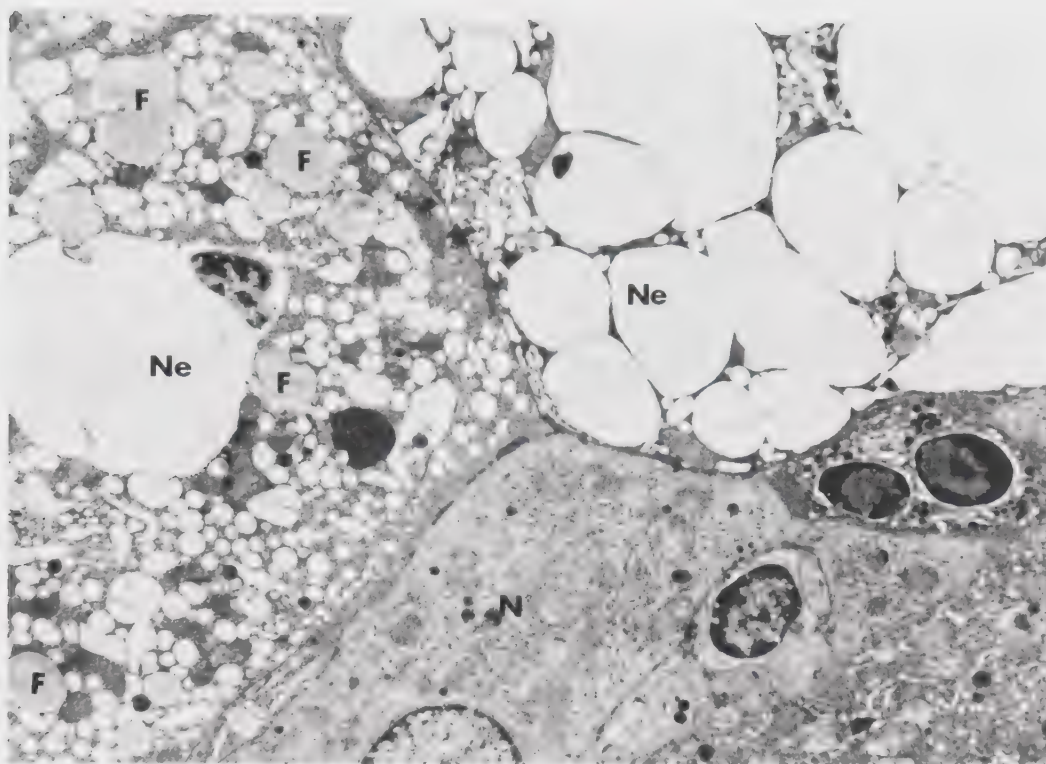Plate 4: PB-treated liver. X 3,500.

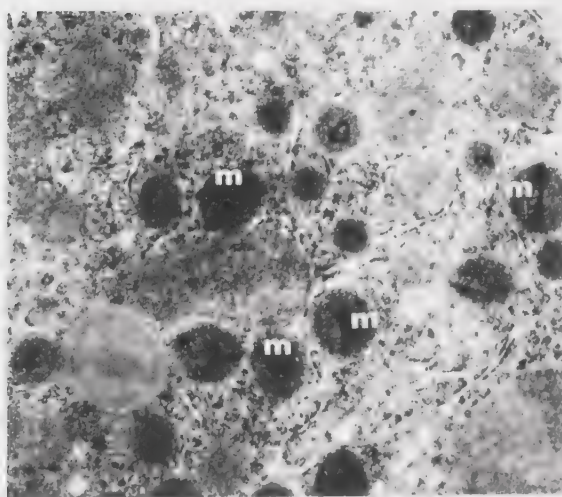Plate 5: PB-CCl₄ liver. X 33,400.

Plate 6: PB-CCl$_4$ liver. X 5,500.

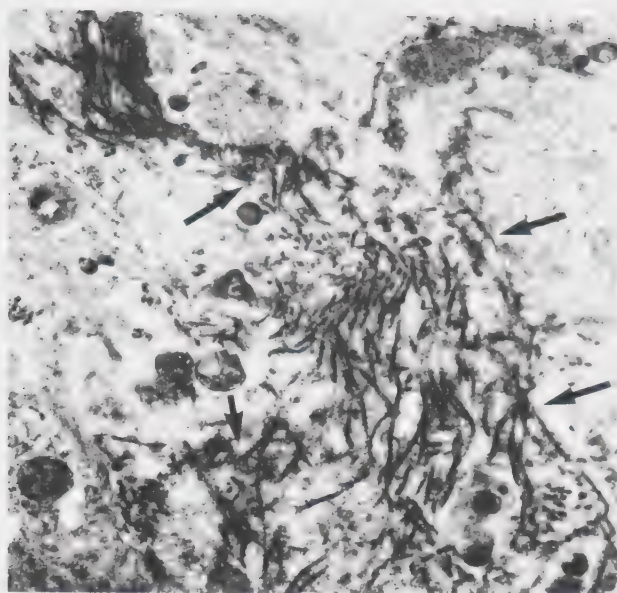Plate 7: PB-CCl$_4$ liver. X 16,700.

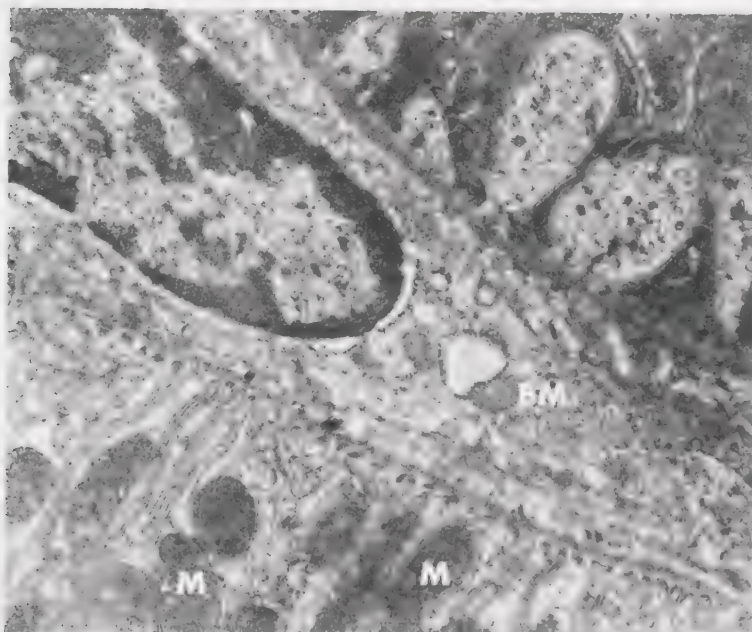Plate 8: PB-CCl$_4$ liver. X 16,700.

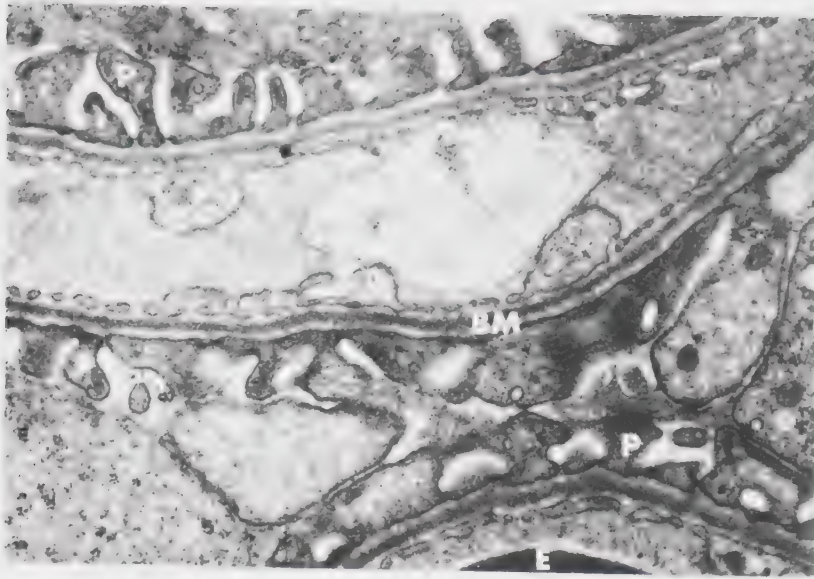Plate 9: Proximal tubule cells of a normal mouse. X 18,900.

Plate 10: Control glomerulus. X 24,600.
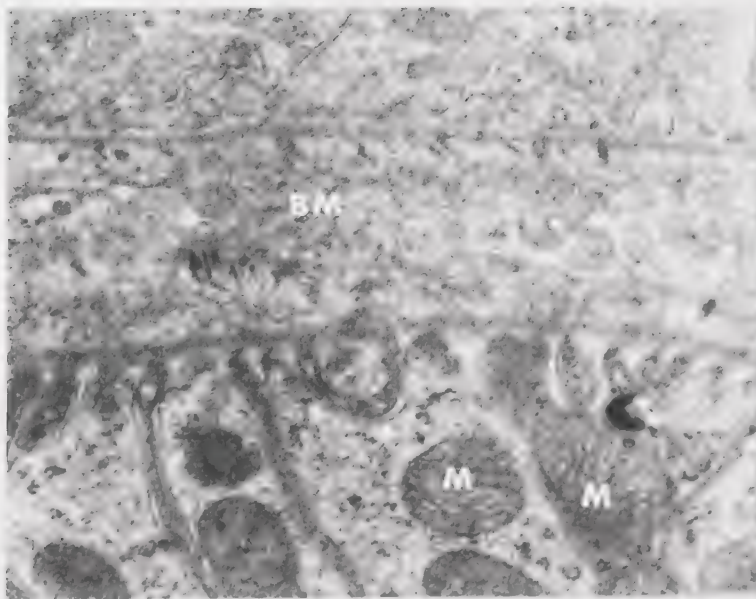

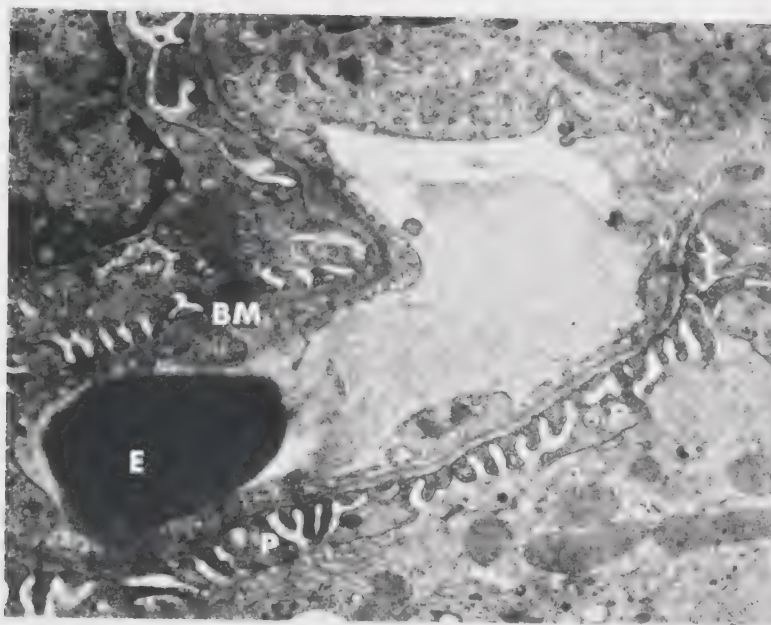Plate 11: Tubular cells of PB-treated kidney. X 28,900.

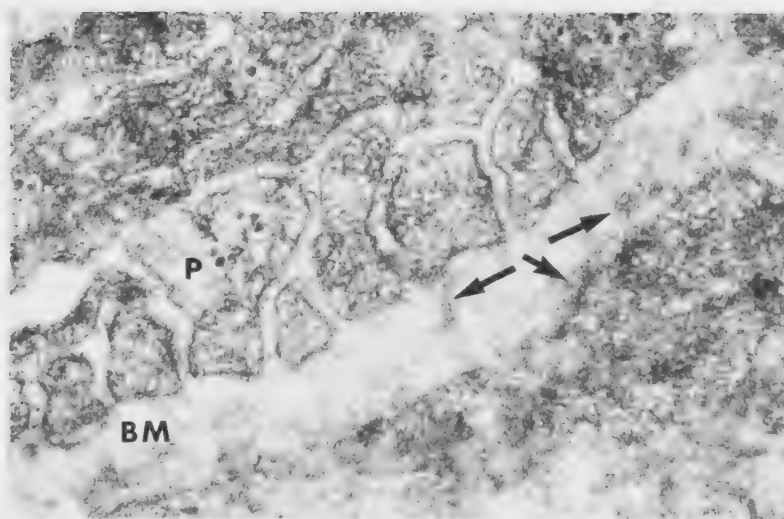Plate 12: Glomerular apparatus of PB-treated kidney. X 12,000.

Plate 13: PB-CCl$_4$ kidney. X 48,000.
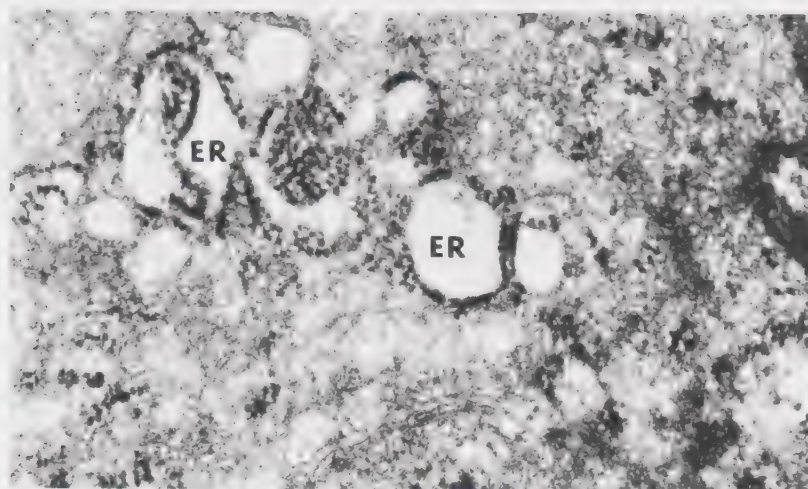
Plate 14: PB-CCl$_4$ kidney. X 48,000.

Definite ultrastructural alterations are observed in the PB-
treated hepatocytes. In Plate 2, the mitochondria (M) appear to be
swollen. There is also proliferation of the SER, some of which also
exhibits dilatation of the cisternae (arrows). Dilatation (arrows),
observed in both RER and SER, becomes pronounced in Plate 3. Fatty
infiltration (arrows) is the third phenomenon observed in this group
of animals (Plate 4).

Some of the actions of PB-CCl$_4$ are illustrated in Plates 5 to
8. There is induced swelling of mitochondria (M) and ER (arrows)
(Plate 5), fatty infiltration (F) of the hepatocytes (Plate 6) and
separation of the nuclear membrane. Plate 6, showing normal intact
cells (N) neighbouring necrotic ones (Ne), illustrates the varying
sensitivities of hepatocytes towards the toxin. A high concentration
of microbodies (m) in Plate 7 is an indication of the hepatic re-
generation process following a reversible injury (151). Fibrosis, as
a result of induced fibrogenetic activity of the perisinusoidal cells

(84) is evident in Plate 8. The disorderly array of collagen fibrils (arrows) is believed to be a response action towards acute liver injury (84, 134).

Plate 9 is an electron micrograph of the proximal tubular cells showing the basement membrane (BM) and the elongated mitochondria (M) which are oriented with their long axis perpendicular to the basement membrane. The glomerular apparatus capillary wall is pictured in Plate 10. To the bottom of the micrograph is an erythrocyte (E). The basement membrane (BM) is characteristically triple-layered. Attached to the basement membrane are many pedicels (P) or foot processes.

The ultrastructures observes in the phenobarbital group (Plates 11 and 12) are not unlike those of the control animals. The basement membrane (BM) is intact and the mitochondria (M) are normal.

The deleterious action of PB-CCl$_4$ treatment in the kidney is demonstrated in Plates 13 and 14. The action can be described as necrotising. There is evidence of loss of homogeneity and mottling of the basement membrane (BM) (Plate 13). Ballooning of the ER is another observation (Plate 14).

DISCUSSION

Great variation in the number of major metabolites in each group has been found. Using TLC-autoradiography, 7, 4 and 3 radio-active metabolites were detected in the control, PB and PB-CCl$_4$ groups respectively. The presence of amphetamine in the urine of all three groups was confirmed by a combined TLC-GLC technique. p-Hydroxyamphetamine was also detected in the urine of the PB-CCl$_4$ animals by GLC, and a small peak with retention time similar to that of standard p-hydroxyamphetamine was also observed in the other two groups. However, the smallness of the peak precluded any confirmatory attempts.

Using a combined preparative TLC-GLC technique, the following compounds were detected in trace quantities in the urine of cirrhotic animals: phenylacetone, benzoic acid and hippuric acid. Although the pooled urine had been subjected to preparative TLC separation and strip extraction prior to introduction into the GC, the resultant gas chromatogram was extremely complicated, thus discouraging further confirmation tests.

The drastic reduction in the number of metabolites from 7 in the control group to 4 and 3 in the PB and PB-CCl$_4$ groups respectively, is proof of a malfunction of the drug-metabolising enzyme system. Metabolic transformation of drugs is achieved by enzymes which alter the compound from a lipid-soluble form to one that is more polar and thus more readily excreted in the urine or the bile. Since the main site of metabolism of xenobiotics is the hepatic SER, in which most of the intrahepatic enzymes are located, cirrhosis of the liver might be expected to produce impaired drug metabolism and diminution in drug tolerance. Impaired metabolism of phenylbutazone and isoniazid has

been reported (74). A prolonged elevation of the plasma level of

chloramphenicol (69) is another example. However, the results of

studies by Brodie et al. (13) of patients with Laennec's cirrhosis

were contradictory to the general belief.

CCl_4 has been cited in many instances to be the agent re-

sponsible for the observed downfall of drug-metabolising activities.

The impaired in vitro metabolism of benzopyrene (87), hexobarbital

(33), aminopyrine (33, 48), p-nitrobenzoic acid (33), ethylmorphine

(116), dimethylaniline (127) and testosterone (94) is evidence of

metabolic derangement. Hexobarbital sleeping time has been reported

to be prolonged in CCl_4-poisoned rats (33). In another study (12), the

in vivo clearance of PB and paraldehyde was delayed. All these ob-

servations are in good accord with the findings of the present inves-

tigation in which the amount of unchanged amphetamine excreted rose

from 38% in the control to 51% in the cirrhotic group. The change in

the relative proportions of the metabolites seems to suggest different

sensitivities of different enzyme systems towards the PB-CCl_4 treatment.

Different microsomal enzymes have been reported to react differently

towards CCl_4 (81, 106, 142). Enzymes that are tightly bound to mem-

branes would be expected to suffer a greater and earlier decline in

activity than those loosely bound and in soluble form.

A decrease in portal and total blood flow is not uncommon in

cirrhotic subjects (72). A decline in blood flow could be due to

fibrosis (Plate 8) or the presence of vascular shunts. A reduction in

hepatic perfusion could lead to hypoxia and other intracellular changes

which could be detrimental to drug uptake, metabolism and clearance.

CCl_4 has been shown to be nephrotoxic in rats (20), rabbits (67) and man (122). However, only one case of renal dysfunction in mice has been reported (144). Male white mice treated with CCl_4 showed a loss of concentration power for p-aminohippuric acid. This biochemical impairment complements the present ultrastructural observations of necrosis, loss of homogeneity and mottling (Plate 13) of the basement membrane and ballooning of the ER (Plate 14)

The pathogenesis of the observed subcellular renal changes may be similar to that of hepatic lesions. An electron-transport system, similar to the one in the liver, is present in rat renal microsomes (20). CCl_4 forms a type II binding spectrum with renal cytochrome P-450, in contrast to a type I spectrum in the liver (20). Different suspectibility of the renal tissues and/or higher endogenous anti-oxidants (20) might require a longer exposure and higher dose for the development of observable lesions.

Mondon et al. (90) suggested that functional integrity of the kidney, and a normal liver, were inseparable. Renal defects are common occurrences among cirrhotics. Renal disturbances have been attributed to haemodynamic changes (65) common among patients of diseased liver. A reduction in renal blood flow has been associated with increased renal vascular resistance and a subsequent fall in glomerular filtration rate (120).

Defect in urinary acidification is another symptom of cirrhosis (10). Excessive tubular re-absorption of sodium ions in cirrhotic patients deprives distal tubules of an adequate level for a proper sodium-hydrogen ion exchange process. This could explain the

initial high pH of the cirrhotic 24 hour urine. Amphetamine, being a weak base, would exist mainly as the free base subject to active re-absorption. It can be assumed that the recycled amphetamine could undergo further metabolism in the liver, producing more acidic metabolites which could be readily excreted. This may account for the delayed excretion by the cirrhotic group.

Filtration is a function of the glomerular apparatus. Increased filtration of protein across the basement membrane is evident in Plate 13; the deposits of electron-dense materials are believed to be protein in nature. However, the fusion of the foot processes, generally associated with increased passage of protein through the basement membrane, is not observed. Focal splitting of the basement membrane of the tubules is further proof of renal damage.

Most of the reported work describing the action of PB on drug metabolism is the short-term induction type. Its effect has been described as enhancing (1, 14, 26, 58, 77, 89). The only negative effect of PB was reported by Sorrell et al. (132) who indicated that the drug was responsible for the observed elevated hepatic levels of triglyceride, phospholipid and total cholesterol. Our own electron microscopic study revealed an increase in fatty deposition (Plate 4), and dilatation of the ER (Plate 3) and the mitochondria (Plate 2). A decrease in metabolic activity, along with impaired uptake and prolonged storage may well be the contributing factors towards the delayed excretion among this group of animals.

PB-dependent induction has been suggested to be both quantitative and qualitative for hepatic microsomal aniline hydroxylase (55). Aniline is classified as a type II compound, a group to

which amphetamine also belongs (79). Whether the metabolism of amphetamine was qualitatively affected in the investigation is not immediately clear, but the possibility does exist.

Biliary excretion is a minor pathway in amphetamine metabolism by mice. PB treatment increases bile flow (37), urinary d-glucaric acid (62) and glucuronyl transferase activity (11) in short-term studies. In liver disease, the relative concentration of the different bile acids is altered (70). The significance of this ob-servation is difficult to apply to the excretion data presented elsewhere in this thesis. One explanation of altered excretion by this mechanism could be an altered intestinal bacterial flora affecting absorption (70) from the gut after biliary elimination.

The ability of a cirrhotic animal to handle a drug is a sum vector of many factors. A change in blood flow, hepatic uptake, storage, bile flow, bile acid metabolism, renal function, hepatic parenchymal cell damage and the adaptability of organs to a disease state have turned the study of drug metabolism in cirrhotic patients into a very complicated picture. Increased sensitivity and prolonged accumulation of drugs inside the body, because of defective metabolism and excretion necessitate the careful prescription of medication.

SUMMARY AND CONCLUSION

From the experimental observations in the present investigation
it can be concluded that:

1. Hepatic cirrhosis was induced in ALAS strain mice by a
    combination of treatment of PB and CCl_4.

2. PB caused ultrastructural changes in the liver but not
    the kidney. Combined treatment with PB–CCl_4 caused
    hepatic changes and had a necrotising effect on renal
    tissues.

3. The percent of the administered dose excreted in urine
    in 72 hours were 84.5, 61.5 and 72.3 respectively for
    the control, PB and PB–CCl_4 groups. Fecal elimination
    in 72 hours was 1.2%, 2.0% and 2.4% of the administered
    dose for these respective groups.

4. The number of radioactive metabolites detected was 7,
    4 and 3 in the urine of the control, PB and PB–CCl_4
    groups respectively.

5. The major urinary constituents in the three groups
    were those listed in Table 21.

6. The qualitative and quantitative metabolism of amphetamine
    is altered by treatment with PB and PB–CCl_4.

Table 21: Major Urinary Metabolites of Amphetamine

Group	No. of Metabolites[a]	Compound	Means of Identification	% of dose
Control	7	Amphetamine	TLC-AutoR[b], GLC	31.8
		p-Hydroxyamphetamine	GLC[c]	
		N-Acetylamphetamine	TLC-AutoR[c]	
		Hippuric acid	TLC-AutoR[c]	
PB	4	Amphetamine	TLC-AutoR, GLC	31.4
		p-Hydroxyamphetamine	GLC[c]	
		N-Acetylamphetamine	TLC-AutoR[c]	
		Hippuric acid	TLC-AutoR[c]	
PB-CCl$_4$	3	Amphetamine	TLC-AutoR, GLC	36.7
		p-Hydroxyamphetamine	GLC	
		N-Acetylamphetamine	TLC-AutoR[c]	
		Hippuric acid	TLC-AutoR[c], GLC[c]	
		Phenylacetone	GLC[c]	
		Benzoic acid	GLC[c]	

[a] detected by TLC-autoradiography

[b] TLC-AutoR=Thin-Layer Chromatography-Autoradiography

[c] positive identification not obtained

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